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(54) Title: NEW METHODS AND COMPOUNDS FOR THE SELECTIVE MODULATION OF ANTIGEN-SPECIFIC T-CELL RESPONSIVENESS (57) Abstract <p>The present invention relates to new FcγRII bridging compositions for impairing the capacity of antigen presenting cells (APCs) to stimulate the activation of antigen-specific T-cells, resulting in modulation of antigen-specific T-cell responsiveness. More particularly, said FcγRII bridging agents are chosen from the group consisting of: aggregated human IgG molecules; aggregated Fc fragments of human IgG molecules; a bivalent monoclonal antibody to the FcγRII; a multivalent monoclonal antibody to the FcγRII; a functionally active fragment of said bivalent or multivalent monoclonal antibody; a recombinant fusion protein of 2 or more human IgG Fc parts; liposome vesicles comprising any of the foregoing, provided that said FcγRII composition prevents the expression of the co-stimulatory molecules B7-1/2 and/or down modulates the ICAM-3 molecule expression by these professional APCs. The present invention also relates to prophylactic and therapeutic methods and compositions to prevent or treat the rejection of solid organs, tissues and cells after transplantation; for inducing T-cell anergy or T-cell tolerance; for treating allergic diseases; or for the treatment of autoimmune diseases. The present invention also relates to FcγRII bridged professional APCs prepared by bridging APCs with an FcγRII bridging agent according to the present invention.</p>		

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**NEW METHODS AND COMPOUNDS FOR THE SELECTIVE MODULATION OF
ANTIGEN-SPECIFIC T-CELL RESPONSIVENESS**

Field of the Invention

5 The present invention relates to the new finding that the bridging of Fc γ type II
receptors on professional antigen presenting cells (APCs) impairs the expression of the
essential co-stimulatory molecules B7-1/2 and causes the down modulation of the adhesion
molecule ICAM-3 expression, resulting in the modulation of antigen-specific T-cell
unresponsiveness. The B7 molecules on APCs provide the essential co-stimulatory signal that
10 determines whether TcR/CD3 signaling after activation of T cells with the specific antigen
leads to full T-cell activation or T cell anergy. The ICAM-3 molecule mediates cellular
interactions of T cells and other lymphocytes at sites of inflammation and specific immune
responses.

The present invention also relates to prophylactic and therapeutic methods to prevent
or to treat allergic diseases.

15 The present invention further relates to the treatment of T-cell mediated autoimmune
diseases.

The present invention also relates to prophylactic and therapeutic methods to prevent
or to treat the rejection of solid organs or cells after allogeneic or xenogeneic transplantation.

Background art

20 A. **Fc-Receptors**

Fc γ -receptors play important roles in several immunological processes such as
phagocytosis of opsonized particulate antigens, clearance of immune complexes, antibody-
mediated cellular cytotoxicity, production of inflammatory mediators, and regulation of
immunoglobulin synthesis (Mellman et al., *J. Cell. Biol.* 96:887 (1983); Kurlander et al.,
25 *J. Immunol.* 133:855 (1984); Shen et al., *J. Immunol.* 137:945 (1986); Graziano and Fanger,

J. Immunol. 138:945 (1987); Zheng et al., *Eur. J. Immunol.* 23:2826 (1993)).

The Fc γ -receptors on human leukocytes can be divided in three major classes, based on their molecular mass, specificity and affinity for IgG, cellular distribution, and reactivity with monoclonal antibodies (Van de Winkel et al., *J. Leuk. Biol.* 49:511 (1991); Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457 (1991)). Fc γ RI is a high affinity receptor for monomeric IgG, consists of three Ig-like extracellular domains and is expressed on monocytes and IFN- γ activated neutrophils (Ierino et al., *J. Immunol.* 150:1794 (1993)). Fc γ RII consists of two Ig-like extracellular domains. This receptor binds monomeric IgG only with low affinity, but has a high affinity for complexed IgG (Ierino et al., *J. Immunol.* 150:1794 (1993)). Fc γ RII is the most widely expressed FcR, it is present on monocytes, dendritic cells, granulocytes, platelets and B cells (Anderson and Looney, *Immunology today* 7:264 (1986); Nestle et al., *J. Immunol.* 151:6535 (1993)). Fc γ RIII also contains two Ig-like extracellular domains and also binds monomeric IgG only with low affinity, but has a high affinity for complexed IgG (Ierino et al., *J. Immunol.* 150:1794 (1993)). Fc γ RIII is expressed on neutrophils, eosinophils, a group of cells referred to as L cells which include natural killer cells and large granular lymphocytes, and on macrophages but not on monocytes (Anderson and Looney, *Immunology today* 7:264 (1986)).

B. T-cell activation

Activation of T cells is the result of ligand-receptor interactions. The TcR/CD3 complex has two functions in antigen-induced activation: a recognition function in which a specific antigen is recognized in the context of the appropriate MHC molecule, and a signalling function in which the recognition event is transmitted across the plasma membrane (Weiss and Imboden *Adv. Immunol.* 41:1 (1987)). However, to induce proliferation and maturation into effector cells, T cells need a second signal in addition to the one mediated by the TcR/CD3 complex. This co-stimulatory signal is normally provided by the cell surface of APCs (Springer et al. *Annu. Rev. Immunol.* 5:223 (1987)). Intercellular signaling after TcR/MHC-peptide interaction in the absence of the co-stimulatory signal results in T-cell inactivation in the form of clonal anergy (Mueller et al. *Annu. Rev. Immunol.* 7:445 (1989)). A number of accessory molecules present on the cell surface of T cells with known ligands on the APCs have been implicated in providing the co-stimulatory signal in T-cell activation:

CD2 with its ligand CD58 (LFA-3), CD11a/CD18 (LFA-1) with CD54 (ICAM-1), CD28 with B7, and CD29/CD49d (VLA-4) with VCAM-1 (Selvaraj et al. *Nature* 326:400 (1987); Springer *Nature* 346:425 (1990); Marlin and Springer *Cell* 51:813 (1987); Van Noesel et al. *Nature* 333:850 (1988); Linsley et al. *Proc. Natl. Acad. Sci. USA* 87:5031(1990);
5 Damle et al. *Proc. Natl. Acad. Sci. USA* 88:6403 (1991)).

So far, the best candidate co-stimulatory signal that determines whether TcR-stimulation leads to full T-cell activation, or to T-cell anergy, is generated by interaction of CD28 on the T cells with B7-1/2 on APCs. It has been demonstrated *in vitro* that cross-linking of the CD28 molecule can rescue mouse T-cell clones from becoming anergic
10 (Harding et al. *Nature* 356:607 (1992)). In addition, it has been shown that B7-1 but not ICAM-1 mediated co-stimulation of T cells can prevent the induction of alloantigen-specific tolerance (Vassiliki et al. *J. Exp. Med.* 178:1753 (1993)).

Co-stimulation of T cells with mAb to the TcR/CD3 complex and CD28 results in greatly enhanced activation (Thompson et al. *Proc. Natl. Acad. Sci. USA* 86:1333 (1989)).
15 It has been demonstrated that cross-linking CD28 with mAb can be replaced by B7, a natural ligand for CD28 (Linsley et al. *J. Exp. Med.* 173:721 (1991); Gimmi et al. *Proc. Natl. Acad. Sci. USA* 88:6575 (1991); De Boer et al. *Eur. J. Immunol.* 22:3071 (1992); Van Gool et al. *J. Immunol.* 150:3254 (1993)). The B7-CD28 interaction can result in a strong proliferative (Linsley et al. *J. Exp. Med.* 173:721 (1991); Gimmi et al. *Proc. Natl. Acad. Sci. USA* 88:6575 (1991); De Boer et al. *Eur. J. Immunol.* 22:3071 (1992)) as well as a
20 cytolytic T-cell response (Van Gool et al. *J. Immunol.* 150:3254 (1993)). It has recently been demonstrated that there are at least two B7 molecules that can functionally interact with CD28 (Hathcock et al. *Science* 262:905 (1993); Freeman et al. *Science* 262:907 (1993); Freeman et al. *Science* 262:909 (1993); Azuma et al. *Nature* 366:76 (1993)). B7-1, originally
25 named B7/BB1 is a monomeric transmembrane glycoprotein with an apparent molecular mass of 45-65 KDa and is, like CD28, a member of the immunoglobulin superfamily (Freeman et al. *J. Immunol.* 143: 2714 (1989)). The second B7 molecule, named B70 or B7-2, is a transmembrane glycoprotein with an apparent molecular mass of approximately 70 KDa and is also a member of the immunoglobulin superfamily (Freeman et al. *Science* 262:909 (1993);
30 Azuma et al. *Nature* 366:76 (1993)). It was initially reported that the expression of the B7-1 molecule was restricted to activated B cells and monocytes stimulated with IFN- γ (Freeman et al. *J. Immunol.* 143: 2714 (1989); Freedman et al. *Cell. Immunol.* 137:429 (1991)). More

recently , B7-1 expression has also been found on cultured peripheral blood dendritic cells (Young et al. *J. Clin. Invest.* 90:229 (1992)) and on *in vitro* activated T cells (Azuma et al. *J. Exp. Med.* 177:845 (1993)). *In vivo*, the B7-1 molecule is constitutively expressed on dendritic cells, on monocytes/macrophages in inflammatory lesions, on a subset of B cells in and around germinal centers and, on T cells in chronic inflammatory lesions (Vandenberghe et al. *Int. Immunol.* 5:317 (1993); Barnaba et al. *Eur. J. Immunol.* 24:71 (1994)). The B7-2 molecule seems to have a very similar distribution pattern as B7-1, with the exception that induction of cell-surface expression seems to be faster (Freeman et al. *Science* 262:909 (1993)) and that it seems to be present on freshly isolated monocytes (Azuma et al. *Nature* 366:76 (1993)).

C. T-cell anergy

Incomplete activation of T cells in the absence of the co-stimulatory signals from the APC results in T-cell tolerance or anergy (Mueller et al., *Annu. Rev. Immunol.* 7:445 (1989)). T-cell anergy is characterized by the fact that anergic T cells do not anymore respond to normal activation signals, even in the presence of all the co-stimulatory signals. It has been demonstrated that cross-linking the CD28 molecule on mouse T cells can rescue these cells from becoming anergic when stimulated with chemically modified APC, a situation that otherwise would result in anergy (Harding et al., *Nature* 356:607 (1992)). It has also been shown that B7-1 but not ICAM-1 mediated co-stimulation of human T cells can prevent the induction of alloantigen-specific tolerance (Vassiliki et al., *J. Exp. Med.* 178:1753 (1993)). In addition, it has been shown that the combination of blocking B7-1/CD28-CTLA4 with a human B7-1 specific monoclonal antibody and cyclosporin A can induce alloantigen-specific tolerance (Van Gool et al., *J. Exp. Med.* 179: (1994)), and that blocking B7-1 and B7-2/CD28-CTLA4 with a soluble CTLA4 fusion protein can induce hypo-responsiveness of human T cells when stimulated with alloantigen (Tan et al., *J. Exp. Med.* 177:165 (1993)). Thus the B7/CD28-CTLA4 interaction seems to play an important role in determining whether T-cell stimulation leads to full activation or T-cell anergy.

D. Allergy

Atopic allergic diseases, i.e. asthma, allergic rhinitis, atopic dermatitis, and food and drug allergy, affect at least 20% of the population in the industrialized world and are an important cause of morbidity, and for asthma mortality. The initial factors involved in the allergic reaction are allergen-specific antibodies of the IgE type and mast cells, which express high affinity receptors for IgE and which are widely distributed in tissues that form our protective barrier against the outside such as mucosae. When an antigen (the allergen) enters the human body, binding to and bridging of receptor-bound IgE on the mast cells results in activation of these cells. This activation will result in the release of histamine that triggers an immediate response (within 20 minutes), as well as the release of soluble factors that initiate a local inflammatory response. This results in the influx of inflammatory cells that give subsequently rise to the late phase (6-24 hours) of the allergic response and to the chronic inflammation seen at places of repeated exposure to allergen. This late phase reaction is characterized by the occurrence of eosinophils.

Thus allergy is characterized by immediate and late phase hypersensitivity reactions to allergens. These immune reactions are associated with elevated serum levels of allergen-specific IgE and to eosinophilia, respectively. Both IgE production and eosinophil production are controlled by T helper (Th) lymphocytes and evidence is accumulating that the aberrant immunological characteristics of atopic allergy can be explained by the hyperactivation of a particular subset of Th cells (Th2).

Helper T cells regulate immune responses via cytokines that they produce upon recognition of specific antigen presented by antigen presenting cells (APC). Individual Th cells (clones) can be distinguished on the basis of the cytokine secretion profile and hence their function (Mosmann et al. *Ann Rev Immunol.* 7:145 (1989)). In response to various antigens, Th cells produce many cytokines simultaneously (type 0 profile). However, in response to intracellular micro-organisms the production of cytokines of the Th cells is biased to high levels of interferon (IFN)- γ and low levels of interleukin (IL)-4 and IL-5 (type 1 cytokine profile, Th1). Such a Th1 response is protective because IFN- γ stimulates the intracellular killing of microbes by phagocytic cells. In contrast, in response to certain helminth types the Th cell response is biased to low levels of IFN- γ and high levels of IL-4 and IL-5 (type 2 cytokine profile, Th2). IL-4 induces B cells to secrete IgE, provided the level of secreted IFN- γ is low, whereas IL-5 promotes the production of eosinophils in the bone marrow. In such cases, the Th2 response is protective, because both IgE and

eosinophils are considered to contribute to the expelling of the helminths.

In vitro studies with allergen-specific Th cell clones prepared from peripheral blood of atopic asthma and eczema patients and from control individuals showed that eosinophilia and elevated levels of allergen-specific IgE are causally related with the occurrence and activation of allergen-specific Th2 cells (Wierenga et al. *J. Immunol.* 144:4651 (1991); Romagnani *Immunol. Today* 13:379 (1993)). The origin of the dominance of these Th2 cells in atopic allergy is unknown. There is accumulating evidence that type 1 and 2 profiles result from modulation of cytokine production (Trinchieri *Immunol. Today* 14:335 (1993); Snijderwint et al. *J. Immunol.* 150:5321 (1993)). Clearly, soluble factors secreted by APC during antigen-presentation are important. APC-derived factors include IL-12 and prostaglandin (PGE)-2, that skew T cell cytokine production towards Th1 and Th2 profiles, respectively. Thus, a low IL-12/PGE-2 production ratio in APC will result in IL-4 dominated T cell responses, whereas a high IL-12/PGE-2 production ratio will result in IFN- γ -dominated T cell responses.

Immunotherapy, which presently consists of the subcutaneous administration of increasing doses of intact or chemically modified allergens, has shown to result in a down-regulation of allergen-induced T-cell proliferation and release of cytokines and histamine-releasing factors by the allergen-specific T cells (Varney et al., *J. Clin. Invest.* 92:644 (1993); Secrist et al., *J. Exp. Med.* 178:2123 (1993)). The exact mechanism of this classic immunotherapy is not known, although it has been reported that this therapy induces a rise in blocking IgG4 antibodies (Van der Zee et al., *J. Immunol.* 137:3566 (1986); Reid et al., *J. Allergy Clin. Immunol.* 78:590 (1986); Bousquet et al., *J. Allergy Clin. Immunol.* 99:43 (1991)). Other reports have demonstrated that CD8+ suppressor T cells are generated, which modulate allergen-specific IgE production (Rocklin et al., *N. Engl. J. Med.* 302:1213 (1980)).

More recently, attempts to specifically inactivate allergen-specific T cells have been undertaken using specific peptides of allergens. The basis for this experimental immunotherapy is the observation that peptides, when given to T cells in the absence of APCs *in vitro* will result in the antigen-specific inactivation (anergy) of the T cells recognizing the particular peptides (Higgins et al., *J. Allergy Clin. Immunol.* 90:749 (1992)). Subcutaneous injection of a specific peptide from the major allergen of cat hair and dander Fel-d1, in mice previously immunized with whole Fel-d1 in Freund's complete

adjuvant resulted in tolerization of T cells (Brinner et al., *Proc. Natl. Acad. Sci. USA* 90:7608 (1993)) It is hypothesized that the mechanism of this inactivation is based on the engagement of the T-cell receptor by the antigen in the absence of the appropriate co-stimulatory signal (O'Hehir et al., *Annu Rev Immunol.* 9:67 (1991)). This co-stimulatory
5 signal is normally provided by the interaction of the B7 molecule on the APCs with the CD28 molecule of the T cells (Harding et al. *Nature* 356:607 (1992); Vassiliki et al. *J. Exp. Med.* 178:1753 (1993)).

E. Autoimmune diseases

A number of studies indicate that costimulation through CD28 ligation might be the
10 initiating event in autoimmunity. The potential of both a primary signal via the TcR and B7 as a costimulatory signal for the generation of autoimmune diabetes has clearly been proven with transgenic mice (Guerder et al., *Immunity* 1:155 (1994); Harlan et al., *PNAS* 91:3137 (1994)). In these studies, it is hypothesized that tolerance to peripheral antigens is induced
15 by triggering the TcR in the absence of essential costimulatory signals. Mice expressing both B7 and a high level of primary antigens (MHC molecules or viral glycoproteins) on pancreatic beta cells developed autoimmune diabetes. The critical role of the absence of B7-mediated costimulation in the induction and maintenance of tolerance to peripheral antigens, and of the B7-mediated signalling in the breakdown of T-cell non-responsiveness, causing
autoimmunity, was obvious.

20 The role of the B7-CD28 interaction in the chronic activation state of T cells, which have been implicated in autoimmune diseases, has been strongly suggested in various studies. Using immunohistochemical techniques, strong B7 expression has been found in lesions of autoimmune diseases, such as rheumatoid arthritis and psoriasis. Furthermore, it has been demonstrated that blocking B7-CD28 interaction could block autoantibody production and
25 prolongation of life in a murine model of autoimmune disease that closely resembles systemic lupus erythematosus in humans (Finck et al., *Science* 265:1225 (1994)).

F. Transplant Rejection

Incompatibility for the histocompatibility antigens, both major (MHC) and minor

antigens, is the cause for graft rejection. Both CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (CTL) are involved in the rejection process. Activation of T cells after transplantation is the result of ligand-receptor interactions, when the TcR/CD3 complex recognizes its specific alloantigen in the context of the appropriate MHC molecule. To induce proliferation and maturation into effector cells, T cells need a second signal in addition to the one mediated by the TcR/CD3 complex. Intercellular signaling after TcR/MHC-peptide interaction in the absence of the costimulatory signal results in T-cell inactivation in the form of clonal anergy (Mueller et al. *Annu. Rev. Immunol.* 7:445 (1989)). It has been demonstrated that blocking B7-1/2, when combined with a donor-specific cell transfusion, can prevent the rejection of MHC-mismatched cardiac allografts in a rat model (Lin et al. *J. Exp. Med.* 178:1801 (1993)). In addition, it has been demonstrated that co-stimulation of T cell by cross-linking the CD28 molecule is resistant to the inhibitory activity of the immunosuppressive drug cyclosporin A (June et al. *Immunol. Today* 11:211 (1990)). This demonstrates the importance of the B7/CD28 interaction in the rejection of transplants.

AIMS OF THE INVENTION

Activation of a certain type of Fc-receptors is reported to occur only when several Fc regions within an antigen-antibody complex simultaneously bind to several Fc-receptors, causing them to be cross-linked (US Patent No. 4,753,927). Such Fc-receptor cross-linking by several Fc regions appears to be the critical signal required to activate certain types of Fc-receptors. The activation of lymphocytes via Fc-receptors is generally viewed as a strong pro-inflammatory event. Inhibition of the immune system functions is reported by these authors to occur if an active site peptide binds to and blocks such an Fc-receptor, and thus prevents Fc regions within an antigen-antibody complex from binding to the receptor, thus blocking Fc-receptor activation.

Although it is generally believed that preventing signal transduction via Fc-receptors is beneficial during various types of inflammatory reactions, the present invention demonstrates that induction of specific signal transduction via Fc-receptor bridging is able

to modulate T-cell responsiveness and can thus be viewed as an anti-inflammatory action.

It is a primary aim of the present invention to provide new therapeutic and/or prophylactic uses for Fc γ RII (CD32) bridging compositions for treating T-cell mediated diseases, more particularly new therapeutic uses for modulation of antigen-specific T-cell responsiveness.

More particularly, it is an aim of the present invention to provide new prophylactic and therapeutic uses for Fc γ RII (CD32) bridging compositions for treating allergic diseases.

More particularly, it is an aim of the present invention to provide new therapeutic and/or prophylactic uses for Fc γ RII (CD32) bridging compositions for treating autoimmune diseases.

More particularly, it is an aim of the present invention to provide new therapeutic and/or prophylactic uses for Fc γ RII (CD32) bridging compositions for preventing rejection of solid organs, tissues or cells after transplantation.

More particularly, it is an aim of the present invention to provide new therapeutic and/or prophylactic uses for Fc γ RII (CD32) bridging agents/compositions for *in vitro* bridging of graft donor professional APCs.

It is particularly also an aim of the present invention to provide and use new Fc γ RII bridging compositions which are characterized by their specific capacity to impair the activation of antigen-presenting cells (APCs) to stimulate the activation of antigen-specific T-cells, resulting in modulation of T-cell responsiveness.

It is a further aim of the present invention to provide and use Fc γ RII bridging compositions which are characterized in that they either (i) prevent the up-regulation (or prevent the expression or down-modulate the expression) of the co-stimulatory B7 molecules on professional APCs, and/or, (ii) they down-modulate (or impair the expression of or prevent the up-regulation of) the adhesion molecule ICAM-3 on professional APCs.

It is a further aim of the present invention to provide methods for preparing such new Fc γ RII bridging compositions.

It is another aim of the present invention to provide methods for the preparation of new therapeutic and/or prophylactic compositions comprising any of said Fc γ RII bridging agents as an active principle.

It is also an aim of the present invention to provide and use Fc γ RII bridged donor professional APCs, or therapeutic compositions comprising the same, for preventing or

treating the rejection of donor grafts or transplants.

It is also an aim of the present invention to provide a method for preparing said Fc γ RII bridged donor professional APCs.

5 It is finally an aim of the present invention to provide an *in vitro* method for screening for or selecting new Fc γ RII bridging agents or compositions being characterized in that they impair the expression of B7 molecules and/or ICAM-3 molecules on professional APC's, resulting in modulation of T-cell responsiveness.

All the aims of the present invention are achieved by the specific embodiments of the invention as detailed below.

10

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in essence on the new finding that bridging several Fc γ RII (CD32) molecules on profesional antigen presenting cells (APCs) prevents the up-regulation of the essential co-stimulatory molecules B7-1/2 and causes the down modulation of the adhesion molecule ICAM-3, resulting in the modulation of antigen-specific T cell
15 unresponsiveness.

It should be evident to the reader that by means of this new finding, the skilled man is put in the ability to screen for or select any new Fc γ RII bridging agent or composition designed to modulate antigen-specific T-cell responsiveness, or any associated therapeutic use as detailed below.

20

The present invention thus relates more particularly to an Fc γ RII (CD32) bridging composition characterized in that it impaires (for impairing) the capacity of antigen presenting cells (APCs) to stimulate the activation of antigen-specific T-cells, resulting in modulation of T-cell responsiveness.

25 The expression "Fc γ RII (CD32) bridging" within the context of the present invention refers to the fact that incubation of professional APCs carrying the Fc γ RII (CD32) with a suitable bridging agent results in the prevention of the up-regulation of the essential co-stimulatory molecules B7-1/2 by these professional APCs and/or results in the down modulation of the adhesion molecule ICAM-3 by these professional APCs. The suitable bridging agents according to the present invention are listed below. Most preferably several
30 Fc γ RII (CD32) molecules are bridged by several Fc regions.

The terms "bridging" or "bridged" used in the present invention have the same meaning as "cross-linking" or "cross-linked". The term cross-linking is preferably not used further in the specification to avoid confusion with the strict meaning of the term "cross-linking" to couple 2 or more protein molecules together, for instance to obtain a state of aggregation of the bridging agent as is explained further.

The expression "prevention of the up-regulation of B7-1/2 molecules" on professional APC's refers to the impaired expression of B7-1/2 molecules as detailed in the Examples section upon applying certain Fc γ RII bridging agents. The expression of B7-1/2 molecules may be measured by analyses such as shown in the Examples section (e.g. FACS analysis) or by means of any other technique known in the art or as indicated below.

The expression "down-modulation of ICAM-3" refers to the impaired expression of ICAM-3 molecules upon applying certain Fc γ RII bridging agents or methods such as described in the Examples section or as indicated below. The expression of ICAM-3 may be measured by analysis methods such as shown in the Examples section, as indicated below or by any other technique known in the art.

The term "professional APCs" is reviewed below.

The terms "Fc γ RII (CD32)", "B7-1/2" and "ICAM-3" are reviewed above.

The Fc γ RII bridging agents/compositions of the present invention aim upon binding to the Fc γ RII on professional APCs and activating this receptor in such a way that the up-regulation of B7-1/2 co-stimulatory molecules is prevented and/or that of the adhesion molecule ICAM-3 is down-modulated resulting in an impaired capacity of the APCs to induce T-cell activation and thus resulting in modulation of T-cell responsiveness. Such Fc γ RII bridging compositions according to the present invention should comprise at least one of the bridging agents as an active principle, with said agents being chosen from the group consisting of:

- aggregated human IgG molecules;
- aggregated Fc fragments of human IgG molecules;
- a bivalent monoclonal antibody to the Fc γ RII;
- a multivalent monoclonal antibody to the Fc γ RII;
- any functionally active fragment of said bivalent or multivalent monoclonal antibody to the Fc γ RII;
- a recombinant fusion protein of 2 or more human IgG Fc parts;

- a liposomal vesicle containing any of the foregoing agents as detailed below.

A selected agent may be confirmed as being a proper Fc γ RII bridging agent according to the present invention by means of the following test system:

5 The amount of B7-1, B7-2 and/or ICAM-3 expressed is measured by any of the techniques known in the art (such as ELISA or immunofluorescence (FACS) analysis in combination with suitable monoclonal antibodies or ligand antigens as described in the Examples section) on the cell surface of professional antigen presenting cells (or APCs, e.g. monocytes cultured in the presence of IFN- γ or GM-CSF) in the presence of the Fc γ RII bridging agent to be tested in comparison to appropriate control conditions and/or agents.

10 Strictly speaking, the terms "agent" and "composition" within the meaning of the present invention differ in that a composition should comprise at least one FcR-bridging agent as an active principle in addition to other component(s). The preferred composition or formulation of said compositions of the invention are detailed extensively below.

15 The term "aggregated" may mean aggregated by means of any chemical cross-linking agent known in the art or aggregated by any other means such by immobilizing the IgG's or fragments thereof to a solid phase as is explained below and in the examples section.

20 "Monoclonal antibodies to the Fc γ RII" refer to monoclonal antibodies which are specifically directed to the Fc γ RII. These monoclonal antibodies can be prepared as described in international application WO 88/00052 (describing the production of monoclonal antibodies specifically directed to the Fc γ RI) or Anderson et al. ((1986) *J. Biol. Chem.* 261:12856). It should, however, be stressed that the Fc γ RII monoclonal antibodies according to the present invention should bind to a site on the Fc γ type II receptor (CD32) such that it prevents the up-regulation of B7-1/2 and/or causes the down-modulation of ICAM-3 by the antigen presenting cells incubated with said Fc γ RII monoclonal antibodies. The selection of such
25 monoclonal antibodies can be performed by measuring the amount of B7-1, B7-2 and/or ICAM-3 produced by professional antigen presenting cells (e.g. monocytes cultured in the presence of IFN- γ or GM-CSF) in the presence of the monoclonal antibodies to be tested in comparison to control monoclonal antibodies by any of the techniques known in the art (e.g. by ELISA or immunofluorescence analysis as described in the Examples section or as
30 described above).

"Bivalent or multivalent monoclonal antibodies to the Fc γ RII", in particular of the IgA or IgM isotype, can be prepared as described in international application WO 88/00052

where the supernatants of the hybridoma clones are directly screened for the production of specific antibodies of the IgM or IgA isotype using specific reagents. Alternatively, IgA secreting hybridoma cells can be obtained from an IgG secreting hybridoma cell line by limiting dilution cloning and selection of Ig-isotype switch variants by methods known to those skilled in the art. In addition, Fc γ RII-specific antibodies of the IgA or IgM isotype can be prepared using recombinant DNA technology by expressing the antigen-binding variable region of the said antibodies in a vector containing the cDNA encoding for the IgA or IgM constant region by methods known to those skilled in the art. These constant regions can be of mouse origin, but more preferably are of primate or human origin. Bivalent or multivalent monoclonal antibodies to Fc γ RII or functionally active fragments (such as F(ab')₂ fragments) of such antibodies can also be prepared by conjugating antibodies with known coupling or cross-linking agents such as protein A, carbodiimide, N-succinimidyl-2(2-pyridylthio) propionate (SPDP) Karpovsky et al. (1984) *J. Exp. Med.* 160:1686; Liu et al (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82:8648). Alternatively, such fragments can be generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases.

The expression "functionally active fragment of said bivalent or multivalent antibody to the Fc γ RII" may refer to an F(ab')₂ fragment of said antibody or conjugated Fab fragments of said multivalent or bivalent monoclonal antibody(ies), provided that the resulting antibody fragments have the effect of preventing the up-regulation of B7-1/2 and/or cause the down-modulation of ICAM-3 expression by the APCs incubated with said antibodies as described above. Conjugated antibody fragments may be obtained as detailed below.

The expression "a liposome vesicle comprising any of the foregoing agents" refers more particularly to liposome vesicles having Fc regions of any of the foregoing bridging agents sticking out of the liposome. Liposome vesicles according to this aspect of the invention may be prepared by any method for preparing liposomes known to the man skilled in the art such as described by A. Gabizon et al., *Cancer Research* 42:4734 (1982); D.S. Cafiso, *Biochem Biophys Acta* 649:129 (1981) and F. Szoka, *Ann Rev Biophys Eng* 9:467 (1980). Other drug delivery systems known in the art may also be applicable and are described in e.g. M.J. Poznansky et al., "Drug Delivery Systems" (R.L. Juliano, ed., Oxford, N.Y., 1980), pp. 253-315; M.L. Poznansky, *Pharm Revs* 36:277 (1984).

The present invention further contemplates an Fc γ RII bridging composition as defined

above, further characterized in that said Fc γ RII bridging agent prevents the up-regulation (or impaires the expression) of B7-1/2 molecules by these APCs.

The present invention contemplates also any of the Fc γ RII bridging composition as defined above, further characterized in that said Fc γ RII bridging agent causes the down modulation (or impaires the expression) of ICAM-3 molecules by these APCs.

According to a preferred embodiment, the present invention relates to any composition as defined above, further characterized in that said composition comprises a specific antigen or antigen-complex combined with an agent capable of bridging Fc γ RII molecules as defined above.

The term "combined" refers to any type of combination known in the art, more particularly implies covalently attaching or cross-linking said antigen or antigen-complex to said bridging agent preferentially via chemical cross-linking. Said antigen or antigen-complex may thus be attached or aggregated to said bridging agent by means of any technique known in the art using any type of attaching agent or cross-linking agent known in the art. Liposome vesicles as bridging agents will preferably contain said antigen or antigen-complex inside of the liposome vesicle. Such vesicles may be prepared by any of the techniques known in the art for liposome vesicle preparation.

It is proposed in the context of the present embodiment of the invention, that combining a specific antigen or antigen-complex with an Fc γ RII bridging agent of the invention *in vivo* would result in presentation of antigenic peptides to specific T cells by professional APC's in the absence of the essential co-stimulatory molecules B7-1/2 or in the presence of reduced concentrations of such molecules on the surface of these APC's. This treatment will result in the modulation of only those T-cells able to recognize the specific antigen in question. This finding is of particular importance in the treatment of diseases where T cells play a crucial role in pathology such as allergic and autoimmune diseases.

More particularly, the present invention relates to an Fc γ RII bridging composition as defined above, further characterized in that said specific antigen or antigen-complex causes allergic diseases (often referred to as allergen). Examples of such antigens that can act as allergens in humans are the major allergen of cat hair and dander, house dust mite antigen, pollen antigens, and bee venom.

The present invention also relates to an Fc γ RII bridging composition as defined above, further characterized in that said specific antigen is an alloantigen.

The term "alloantigen" refers to foreign MHC antigens, recognized by specific T cells and responsible for the onset of transplant rejection.

In addition, the present invention relates to an Fc γ RII bridging composition as defined above, further characterized in that said specific antigen is an antigen causing the autoimmune attack on the body's own tissue (often referred to as an autoantigen).

According to yet another preferred embodiment, the present invention relates to any of the compositions as defined above, further characterized in that said Fc γ RII bridging agent consists of aggregated human IgG molecules or aggregated Fc fragments of human IgG molecules.

According to yet another preferred embodiment, the present invention relates to any of the compositions as defined above, further characterized in that said Fc γ RII bridging agent consists of a bivalent or multivalent Fc γ RII specific monoclonal antibody, or functionally active fragments of said Fc γ RII specific monoclonal antibody.

According to yet another preferred embodiment, the present invention relates to any composition as defined above, further characterized in that said Fc γ RII bridging agent consists of a recombinant fusion protein of two or more human IgG Fc parts.

For this purpose, the present invention also relates to a recombinant vector, particularly for cloning and/or expression, with said recombinant vector comprising a vector sequence, an appropriate prokaryotic, eukaryotic or viral promoter sequence followed by the nucleotide sequences comprising the nucleic acid sequence encoding such a recombinant fusion protein comprising at least two human IgG parts, and with said recombinant vector allowing the expression of said recombinant protein as defined above in a prokaryotic, or eukaryotic host or in living mammals when injected as naked DNA.

The term "vector" may comprise a plasmid, a cosmid, a phage, or a virus.

In order to carry out the expression of the polypeptides of the invention according to this aspect of the present invention in bacteria such as *E. coli* or in eukaryotic cells such as in *S. cerevisiae*, or in cultured vertebrate or invertebrate hosts such as insect cells, Chinese Hamster Ovary (CHO), COS, BHK, and MDCK cells, the following steps are carried out:

- transformation of an appropriate cellular host with a recombinant vector, in which a nucleotide sequence coding for a fusion protein of two or more human IgG Fc parts has been inserted under the control of the appropriate regulatory elements, particularly a promoter

recognized by the polymerases of the cellular host and, in the case of a prokaryotic host, an appropriate ribosome binding site (RBS), enabling the expression in said cellular host of said nucleotide sequence. In the case of an eukaryotic host any artificial signal sequence or pre/pro sequence might be provided, or the natural signal sequence might be employed

- culture of said transformed cellular host under conditions enabling the expression of said insert.

According to yet another preferred embodiment, the present invention relates to any of the compositions as defined above, further characterized in that said Fc γ RII bridging agent consists of a liposome vesicle comprising at least one Fc γ RII bridging agents with said agents being chosen from the group consisting of:

- aggregated human IgG molecules;
- aggregated Fc fragments of human IgG molecules;
- a bivalent monoclonal antibody to the Fc γ RII;
- a multivalent monoclonal antibody to the Fc γ RII;
- any functionally active fragment of said bivalent or multivalent monoclonal antibody to the Fc γ RII;
- a recombinant fusion protein of 2 or more human IgG Fc parts;
- a liposome vesicle comprising any of the foregoing agents as detailed above.

For this purpose, the present invention also relates to methods of preparing liposomes containing an Fc γ RII bridging agent as detailed above.

The present invention also contemplates any method for preparing any of the Fc γ RII bridging compositions as defined above.

Examples of the preparation of some of the Fc γ RII bridging agents and compositions according to the present invention are disclosed above, in the Examples section or are within the knowledge of the man skilled in the art.

The present invention relates particularly to the therapeutic uses of any of the above-defined Fc γ RII bridging composition.

It should be stressed that the term "therapy" or "therapeutics" within the concept of the present invention is to be interpreted as including "therapy" or "therapeutics" as such as well as "prophylaxis" or "prophylactics".

Target cells for treatment with the Fc γ RII bridging compositions according to the present invention may include professional antigen presenting cells (APCs) such as human leukocytes, preferably macrophages, monocytes, dendritic cells, Langerhans cells or B cells. These target cells may possibly be activated before or during treatment with e.g. IFN- γ , or GM-CSF. If desired, target cells to be treated may be derived from the donor of a graft in transplantation.

The compositions of this invention will be administrated at a certain concentration that is therapeutically effective for the envisaged treatment.

To accomplish this goal, the composition may be formulated using a variety of acceptable excipients known in the art. Typically, the compositions are administered by injection, either intravenously, intradermally, intramuscular or subcutaneously. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Before administration to patients, formulants may be added to the composition or bridging agents of the invention. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono, di, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having an -OH group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferably between 2.0 and 6.0 w/v%. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the

composition to minimize pH changes in the solution before lyophilization or after reconstitution. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, in case the composition comprises antibodies as an active principle these can be chemically modified by covalent conjugation to a polymer to increase its circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in US Patent Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula : $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al., 1988, *J. Bio. Chem.* 263 : 15064-15070, and a discussion of POG/IL-2 conjugates is found in US Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions

are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

The dosage and mode of administration will depend on the therapeutic purpose. Generally, the compositions are administered so that aggregated IgG antibodies are given at a dose between 1 μ g/kg and 20mg/kg, more preferably between 20 μ g/kg and 10mg/kg, most preferably between 1 and 7mg/kg.

Therapy with the Fc γ RII bridging compositions or the Fc γ RII bridged cells according to the present invention may be performed in conjunction with any other known techniques or compounds for treatment of inflammatory, allergic or autoimmune diseases or for treatment of transplantation rejection, such as for instance immunosuppressive and anti-inflammatory drugs (e.g. cyclosporin A (CsA), methotrexate, prednisolone, dexamethasone, FK506, rapamycin, cyclooxygenase inhibitors such as indomethacin and corticosteroids) or immunomodulatory cytokines (such as IFN- γ , IL-10 or IL-12).

The new Fc γ RII bridging compositions according to the present invention may further also be used for any other human or animal treatment or diagnostic purpose for which they are possibly suited.

According to a particularly preferred embodiment, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above as a medicament, more particularly for treating T-cell mediated diseases, even more particularly for the preparation of a medicament for treating T-cell mediated diseases.

An Fc γ RII bridging composition to be used according to this aspect of the invention comprises particularly new Fc γ RII bridging agents or compositions according to the above specified aspects of the present invention.

According to an even more preferred embodiment, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above, for the modulation of antigen-specific T-cell responsiveness, more particularly for the preparation of a medicament for the modulation of antigen-specific T-cell responsiveness.

Alternatively, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above, for inducing T-cell tolerance or anergy, more particularly for

the preparation of a medicament for inducing T-cell tolerance or anergy.

The terms "T-cell anergy" and "T-cell tolerance" are reviewed above.

The invention provides a method for the induction of antigen-specific T-cell tolerance or T-cell anergy by modulating the co-stimulatory function of professional APCs by providing to a subject in the need of such a treatment an effective amount of a specific antigen combined with an agent capable of bridging the Fc γ RII with at least one of the above-described functional effects of preventing the essential co-stimulatory molecules B7-1 and -2 from being up-regulated and by down-modulating the adhesion molecule ICAM-3. Said Fc γ RII-bridging agent is selected from the group consisting of: aggregated human IgG molecules; aggregated Fc fragments of human IgG molecules; a bivalent monoclonal antibody specific to the Fc γ RII; a functionally active fragment of the latter antibodies; a multivalent monoclonal antibody specific to the Fc γ RII; a recombinant fusion protein of 2 human IgG Fc parts; liposome vesicles containing at least one of the foregoing bridging agents; all as detailed above.

Fc γ RII bridging agents or compositions to be used according to this particular aspect of the present invention may comprise known FcR bridging compositions, agents or principles or in a preferred way specific Fc γ RII bridging compositions particularly aimed at in the present invention (resulting in the prevention of expression of B7 molecules and/or the down-modulation of ICAM-3 molecules expression) as detailed above.

According to a more particular embodiment, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above for treating allergic diseases, more particularly for the preparation of a medicament for treating allergic diseases in humans, such as asthma, allergic rhinitis, atopic dermatitis, food and drug allergy.

The invention also provides a method for treating allergic diseases in humans, wherein said method comprises providing to a subject in the need of such a treatment an effective amount of a specific antigen combined with an agent capable of bridging Fc γ RII molecules with the above-described functional effects of preventing the essential co-stimulatory molecules B7-1 and -2 from being up-regulated and by down-modulating the adhesion molecule ICAM-3, wherein said Fc γ RII-bridging agent is selected from the group consisting of: aggregated human IgG molecules; aggregated Fc fragments of human IgG molecules; a bivalent monoclonal antibody specific to the Fc γ RII; a multivalent monoclonal antibody specific to the Fc γ RII, or functionally active fragments of the latter antibodies; a recombinant

fusion protein of 2 or more human IgG Fc parts; liposomes containing any of the foregoing as detailed above, possibly in combination with an allergen and other immunomodulatory agents as detailed above.

5 Fc γ RII bridging agents or compositions to be used according to this particular aspect of the present invention may comprise known FcR bridging agents or compositions or in a preferred way, specific Fc γ RII bridging agents or compositions particularly aimed at in the present invention (resulting in the prevention of B7-1/2 molecules expression and/or the down-modulation of ICAM-3 molecules expression) as detailed above.

10 According to another particular embodiment, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above, for preventing rejection of solid organs, tissues and cells after transplantations, more particularly for the preparation of a medicament for preventing rejection of solid organs, tissues or cells after transplantation in humans.

15 The invention also provides a method for preventing rejection of solid organs, tissues or cells after transplantation in humans, wherein said method comprises providing to a subject in the need of such a treatment an effective amount of a specific alloantigen combined with an agent capable of bridging Fc γ RII molecules with the above-described functional effects of preventing the essential co-stimulatory molecules B7-1 and -2 expression and by down-modulating the adhesion molecule ICAM-3 expression, wherein said Fc γ RII-bridging agent is selected from the group consisting of: aggregated human IgG molecules; aggregated Fc fragments of human IgG molecules; a bivalent monoclonal antibody to the Fc γ RII; a multivalent monoclonal antibody to the Fc γ RII, or functionally active fragments of the latter antibodies; a recombinant fusion protein of 2 or more human IgG Fc parts; liposomes containing any of foregoing as detailed above, possibly in combination with an alloantigen and an immunosuppressive or immunomodulatory agent as detailed above.

20 Fc γ RII bridging agents or compositions to be used according to this particular aspect of the present invention may comprise known FcR bridging agents or principles or in a preferred way specific Fc γ RII bridging agents or compositions particularly aimed at in the present invention (resulting in the prevention of B7-1/2 molecules expression and/or the down-modulation of ICAM-3 molecules expression) as detailed above.

30 Target cells for treating *in vitro* or *in vivo* with the Fc γ RII bridging agents or compositions according to the present aspect of the invention are preferably professional

APCs treated with any form of aggregated or immobilized human IgG's, or aggregated Fc fragments of human IgG; or soluble human IgG's in the presence of antibodies to human IgG; or in the presence of bivalent or multivalent Fc γ RII monoclonal antibodies, or in the presence of functionally active fragments of the latter antibodies, or in the presence of a recombinant fusion protein of 2 or more human IgG Fc parts, or in the presence of liposomes containing any of the foregoing.

The term "aggregated" may mean aggregated by means of any known chemical cross-linking agent.

The term "immobilized " may refer to any known immobilization method on any known substrate such as a microtiter plate, a membrane (e.g. nylon or nitrocellulose), a microsphere (bead) or inserted into a liposome vesicle. Prior to application to the membrane or fixation it may be convenient to modify the IgG in order to facilitate fixation or improve its binding efficiency to Fc-receptors.

According to yet another particular embodiment, the present invention relates to the use of any of the Fc γ RII bridging compositions as defined above for treating autoimmune diseases, more particularly for the preparation of a medicament for the treatment of autoimmune diseases, such as thyroiditis, rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, autoimmune diabetes.

The invention also provides a method for the treatment of autoimmune diseases, wherein said method comprises providing to a subject in the need of such a treatment an effective amount of a specific autoantigen combined with an agent capable of bridging Fc γ RII molecules with the above-described functional effects of preventing the essential co-stimulatory molecules B7-1 and -2 expression and/or by down-modulating the adhesion molecule ICAM-3 expression, wherein said Fc γ RII-bridging agent is selected from the group consisting of: aggregated human IgG molecules; aggregated Fc fragments of human IgG molecules; a bivalent monoclonal antibody to the Fc γ RII; a multivalent monoclonal antibody to the Fc γ RII; functionally active fragments of the latter antibodies; a recombinant fusion protein of 2 or more human IgG Fc parts; liposomes containing any of the foregoing as detailed above, possibly in combination with an autoantigen and an immunosuppressive or immunomodulatory agent as detailed above.

Fc γ RII bridging agents or compositions to be used according to this particular aspect of the present invention may comprise known FcR bridging agents or principles or in a

preferred way specific Fc γ RII bridging agents or compositions particularly aimed at in the present invention (resulting in the prevention of B7-1/2 molecules expression and/or the down-modulation of ICAM-3 molecules expression) as detailed above.

According to a special embodiment, the present invention relates to a medicament comprising a composition as defined above.

According to a further embodiment, the present invention relates to Fc γ RII bridged professional APCs such as monocytes such as prepared by bridging professional APCs such as monocytes with any of the Fc γ RII bridging compositions as defined above.

The invention also provides bridged professional APCs as defined above for use as a medicament, particularly for preventing a condition as detailed below.

The invention also relates to a method for preparing such bridged APCs as detailed above.

The invention also provides a method for preventing rejection of solid organs, tissues or cells after transplantation in humans, wherein said method comprises providing to a subject in the need of such a treatment an effective amount of alloantigen-expressing professional APCs such as monocytes from the graft donor on which several Fc γ RII molecules have been bridged with the above-described functional effects of preventing the essential co-stimulatory molecules B7-1 and -2 expression and by down-modulating the adhesion molecule ICAM-3 expression, wherein said Fc γ RII-bridging is accomplished by a method selected from the group consisting of: culturing the professional APCs (such as monocytes) in culture dishes coated with human IgG; culturing the APCs in culture dishes together with aggregated human IgG molecules or aggregated Fc fragments of human IgG molecules; culturing the APCs in culture dishes in the presence of soluble human IgG and an antibody to human IgG; culturing the APCs in culture dishes in the presence of a bivalent monoclonal antibody to the Fc γ RII; culturing the APCs in culture dishes in the presence of a multivalent monoclonal antibody to the Fc γ RII, or culturing the APCs in culture dishes in the presence of any functionally active fragment of the latter antibodies; culturing the APCs in culture dishes in the presence of a recombinant fusion protein of 2 or more human IgG Fc parts; liposome vesicles comprising any of the foregoing as detailed above.

Fc γ RII bridging agents or compositions to be used according to this particular aspect of the present invention may comprise known FcR bridging agents or principles or in a preferred way specific Fc γ RII bridging compositions particularly aimed at in the present

invention (resulting in the prevention of B7-1/2 molecules expression and/or the down-modulation of ICAM-3 molecules expression) as detailed above.

The present invention also contemplates a therapeutic composition comprising Fc γ RII bridged professional APCs as defined above.

5 According to another embodiment, the present invention relates to Fc γ RII bridged professional APCs as defined above, for use as a medicament, more particularly a medicament for treating or preventing any of the above-mentioned disease states.

10 More particularly, the present invention relates to the use of Fc γ RII bridged professional APCs as defined above for the preparation of a medicament for preventing rejection of solid organs, tissues or transplantations in humans.

The present invention also relates to an *in vitro* method for screening for or selecting a new Fc γ RII bridging agent or composition, with said bridging agent or composition being characterized as preventing the B7 molecules expression on professional APCs and/or down modulating ICAM-3 expression on professional APCs.

15 A test system to screen for such molecules comprises an *in vitro* experimental set up in which professional APCs (for instance monocytes) are incubated in the absence or presence of the molecule(s) to be tested as having possible Fc γ II bridging capacities as detailed above by any of the techniques known in the art (such as ELISA or immunofluorescence (FACS) analysis as described in the Examples section) and measuring
20 the amount of B7-1, B7-2 and/or ICAM-3 is measured.

BRIEF DESCRIPTION OF THE TABLES AND FIGURES

Table 1 : Modulation of cell surface antigens on monocytes after FcR bridging. Cell surface expression of various antigens was determined after overnight culture on plates coated with human IgG or on un-coated plates as control. The relative expression is defined as the expression after overnight culture on human IgG-coated plates compared to the expression after culture in un-coated plates. Strong increase is indicated by + + +, modest increase by + +, slight increase by +, strong decrease by ---, modest decrease by -- and slight decrease by -. No change in expression is indicated by ○ in the Table. Cell surface expression was measured by FACS analysis using specific monoclonal antibodies to the cell surface molecules as described in the specific Examples. (*) designates that B7-1/2 expression was determined with the CTLA-4Ig fusion protein as described in the specific Examples.

Table 2 : FcR bridging inhibits antigen-specific proliferation of T cells. PBMC (10^6 cells/ml) were cultured in 96-well flat bottom culture plates pre-coated overnight either with human serum albumin (HSA) or with human IgG (HGG). The following antigens were added to the culture : Tetanus toxoid (0.5Lf/ml), Varidase (100IU/ml), Tuberculin (5IU/ml), Cytomegalovirus antigen (CMV) (0.01IU/ml), Herpes simplex virus antigen (0.02IU/ml), Varicella antigen (0.1IU/ml), Mumps antigen (1/2000), Influenza virus antigen (1/1000), Candida albicans (0.5 μ g/ml). After 6 days of culture, cells were pulsed for 8h with 1 μ Ci(3 H)-Thymidine. Proliferative values represent the average of triplicate wells. (+ +): The following mitogens were used as controls : PHA (0.5 μ g/ml), ConA (5 μ g/ml), PWM (0.5 μ g/ml). After 3 days of culture (6 days in the case of PWM), cells were pulsed for 8h with 1 μ Ci(3 H)-Thymidine. Proliferative values represent the average of triplicate wells.

Table 3 : Release of soluble immunosuppressive mediators after FcR bridging. Purified T cells (10^6 cells/ml) were cultured in the presence of syngeneic or allogeneic monocytes (10^6 cells/ml) as stimulator cells. Cultures were set up in normal plates, or plates precoated with human IgG or F(ab')₂ fragments of human IgG. MLR supernatants were taken after 24h culture and tested for the presence of various cytokines. The results are representative of one experiment out of three.

(+ +): TNF- α was determined by ELISA with a detection limit of 10 pg/ml. (§): IL-10 was measured by ELISA with a detection limit of 5 pg/ml. (||): TGF- β was measured in a bioassay using the MV1 Lu cell line. This bioassay has a detection limit of 50 pg/ml. (<) means below the detection limit of the specific bioassay.

5 **Table 4 : PGE2 production by human monocytes after FcR bridging.**

Purified T cells (10^6 /ml) were cultured in the presence of syngeneic or allogeneic monocytes (10^6 /ml) as stimulator cells. After 3 days of culture, cells were pulsed for 16h with 0.5 μ Ci(3 H)-Thymidine. Proliferative values represent the average of triplicate wells. (+ +): After 24h, supernatants were recovered and analyzed for PGE2 content using an ELISA system.

10 (§): Cells were cultured in 96-well round bottom culture plates. (||): Cells were cultured in 96-well round bottom culture plates pre-coated with human IgG. (π): as in (||) but also the cyclooxygenase inhibitor, indomethacin (100 μ M) was added at the beginning of the culture.

Table 5 : FcR bridging on monocytes from an Fc γ RII-deficient individual results in inhibition of antigen-specific proliferation of T cells.

15 PBMC (10^6 cells/ml) from an Fc γ RII-deficient individual were cultured in 96-well flat bottom culture plates pre-coated overnight either with human serum albumin (HSA) or with human IgG (IgG). The following antigens were added to the culture : Tetanus toxoid (0.5Lf/ml). Varidase (100IU/ml), Tuberculin (5IU/ml), Cytomegalovirus antigen (CMV) (0.01IU/ml), Herpes simplex virus antigen (0.02IU/ml), Varicella antigen (0.1IU/ml), Mumps antigen (1/2000), Influenza virus antigen (1/1000), Candida albicans (0.5 μ g/ml). After 6 days of culture, cells were pulsed for 8h with 1 μ Ci(3 H)-Thymidine. Proliferative values represent the average of triplicate wells. (+ +): The following mitogens were used as controls : PHA (0.5 μ g/ml), ConA (5 μ g/ml), PWM (0.5 μ g/ml). After 3 days of culture (6 days in the case of PWM), cells were pulsed for 8h with 1 μ Ci(3 H)-Thymidine. Proliferative values represent the average of triplicate wells.

25 **Figure 1. Modulation of B7-1 expression on human monocytes.** Monocytes (10^6 /ml) were cultured on human IgG-coated dishes or with various cytokines for 24h and analyzed by FACS for B7-1 expression using mAb B7-24. A: as control monocytes were analysed directly after isolation; b: monocytes cultured 24h in medium; c: monocytes cultured 24h on human IgG-coated dishes; d: monocytes cultured 24h with IFN- γ ; e: monocytes cultured 24h

with TNF- α ; f: monocytes cultured 24h with GM-CSF; g: monocytes cultured 24h with IL-2; h: monocytes cultured 24h with IL-4. Staining with isotype-matched antibody is shown as control.

Figure 2. Modulation of B7-1 expression on human monocytes by Fc γ RII bridging.

Monocytes (10^6 /ml) were cultured for 24h in the presence of soluble human IgG (dotted line), rabbit anti-human IgG (solid line) or soluble human IgG and rabbit anti-human IgG (dashed line). B7-1 expression was analyzed by FACS using mAB B7-24.

Figure 3. Effects of FcR bridging on B7-1, CD40, HLA-DR and ICAM-1 expression on monocytes when activated by IFN- γ or GM-CSF.

Monocytes (10^6 /ml) were cultured for 24h in medium; in medium on human IgG-coated dishes; in the presence of GM-CSF; in the presence of IFN- γ ; on human IgG-coated dishes in the presence of IFN- γ . After a 24h culture period, cells were recovered and analyzed by FACS. Staining with isotype-matched antibody is shown as control. The monoclonal antibodies used are as detailed in the Materials and Methods.

Figure 4. FcR bridging modulates both B7-1 and B7-2 on monocytes.

Monocytes (10^6 /ml) were cultured simultaneously on human IgG-coated dishes in the presence of IFN- γ , GM-CSF, or medium alone. After 24h, cells were recovered and analyzed for B7-1 and B7-2 expression by FACS analysis using the CTLA4-Ig fusion protein. As control the staining with the secondary antibody only is shown. A: monocytes cultured 24h in medium; b: monocytes cultured 24h on human IgG-coated dishes; c: monocytes cultured 24h with GM-CSF; d: monocytes cultured 24h with GM-CSF on human IgG-coated dishes; e: monocytes cultured 24h with IFN-; f: monocytes cultured 24h with IFN- on human IgG-coated dishes.

Figure 5A. Effect of FcR bridging on monocytes on the activation of T cells in MLR.

Purified T cells (10^6 /ml) were cultured in 96-well round bottom tissue culture plates (circles) or plates pre-coated with human IgG (triangles) in the presence of various numbers of syngeneic (closed symbols) or allogeneic (open symbols) monocytes as stimulator cells. A: T-cell proliferation was measured after 3 days by [3 H]-Thymidine incorporation and is expressed as the mean of three different experiments \pm SD.

Figure 5B. Effect of FcR bridging on monocytes on the activation of T cells in MLR.

Purified T cells ($10^6/\text{ml}$) were cultured in 96-well round bottom tissue culture plates (circles) or plates pre-coated with human IgG (triangles) in the presence of various numbers of syngeneic (closed symbols) or allogeneic (open symbols) monocytes as stimulator cells. IL-2 production by the T cells was measured after 24 hours and is expressed as the proliferative response of the CTLL bioassay.

Figure 6A. Blocking the B7-1/CD28 interaction is not sufficient to block the activation of T cells in MLR.

Purified T cells ($10^6/\text{ml}$) were cultured in the presence of various numbers of monocytes as stimulator cells. Monocytes were added directly to the T cells (circles) or after pre-incubation with an anti-B7 mAb (triangles). After 3 days of culture T-cell proliferation was measured by [^3H]-Thymidine incorporation and is expressed as the mean of three different experiments \pm SD.

Figure 6B. Blocking the B7-1/CD28 interaction is not sufficient to block the activation of T cells in MLR.

Purified T cells ($10^6/\text{ml}$) were cultured in the presence of various numbers of allogeneic monocytes as stimulator cells. Monocytes were added directly to the T cells (circles) or after pre-incubation with an anti-B7 mAb (triangles). IL-2 production by the T cells was measured after 24 hours and is expressed as the proliferative response of the CTLL bioassay.

Figure 7A. Impairment of T-cell stimulatory capacity of monocytes after FcR bridging with intact human IgG is specifically mediated by Fc γ RII.

Purified T cells ($10^6/\text{ml}$) were cultured in 96-well flat bottom tissue culture plates pre-coated with F(ab')₂ fragments of human IgG (light bars) or plates pre-coated with intact human IgG (dark bars) in the presence of syngeneic monocytes which were pre-incubated with monoclonal antibodies to different Fc-receptors (anti-Fc γ RI mAb 197, anti-Fc γ RII mAb IV.3). T-cell proliferation was measured after 6 days by (^3H)-Thymidine incorporation. T-cell proliferation induced by PWM.

Figure 7B. Impairment of T-cell stimulatory capacity of monocytes after FcR bridging with intact human IgG is specifically mediated by Fc γ RII. Purified T cells (10^6 /ml) were cultured in 96-well flat bottom tissue culture plates pre-coated with F(ab')₂ fragments of human IgG (light bars) or plates pre-coated with intact human IgG (dark bars) in the presence of syngeneic monocytes which were pre-incubated with monoclonal antibodies to different Fc-receptors (anti-Fc γ RI mAb 197, anti-Fc γ RII mAb IV.3). T-cell proliferation was measured after 6 days by (³H)-Thymidine incorporation. T-cell proliferation specific for the Varidase antigen.

Figure 7C. Impairment of T-cell stimulatory capacity of monocytes after FcR bridging with intact human IgG is specifically mediated by Fc γ RII. Purified T cells (10^6 /ml) were cultured in 96-well flat bottom tissue culture plates pre-coated with F(ab')₂ fragments of human IgG (light bars) or plates pre-coated with intact human IgG (dark bars) in the presence of syngeneic monocytes which were pre-incubated with monoclonal antibodies to different Fc-receptors (anti-Fc γ RI mAb 197, anti-Fc γ RII mAb IV.3). T-cell proliferation was measured after 6 days by (³H)-Thymidine incorporation. T-cell proliferation specific for the Herpes antigen.

Figure 8. The impaired capacity of monocytes to stimulate antigen-specific T-cell activation mediated by FcR bridging with intact human IgG can be prevented by mAb to Fc γ RII. Purified T cells were cultured in 96-well flat bottom tissue culture plates pre-coated with human serum albumin (black bars and dotted bars) or plates pre-coated with intact human IgG (squared bars and hatched bars) with syngeneic monocytes in the absence (black bars and squared bars) or presence (dotted bars and hatched bars) of anti-FcRII mAb IV.3. T-cell proliferation was measured after 6 days by ³(H)-Thymidine incorporation.

EXAMPLES

The present invention is based on the new finding that the bridging of low affinity IgG receptor Fc γ RII (CD32) molecules on monocytes specifically prevents the up-regulation of the essential co-stimulatory molecules B7-1/2 expression and results in the down modulation of the adhesion molecule ICAM-3 expression, with the functional consequence of an impaired capacity of the monocytes to co-stimulate the activation of antigen-specific T cells. Such impaired co-stimulatory capacity of monocytes presenting specific antigen to T cells may lead to T-cell unresponsiveness or T-cell tolerance (Harding et al. *Nature* 356:607 (1992); Vassiliki et al. *J. Exp. Med.* 178:1753 (1993)). This T-cell tolerance toward specific antigens is valuable for certain clinical usages in treating T-cell mediated diseases. Although the importance of the B7 molecules in co-stimulation of T cells and the prevention of T-cell tolerance is known in the art and although it is also known that bridging of Fc γ RII results in the activation of monocytes resulting in the release of cytokines and other soluble mediators, nothing in the art relates to the finding that the specific bridging of the low affinity IgG receptor Fc γ RII (CD32) on monocytes very selectively prevents the up-regulation of the essential co-stimulatory molecules B7-1/2 expression and down-modulates the ICAM-3 adhesion molecule expression. The observed modulation of the B7 molecules and the ICAM-3 molecule expression is a specific process in the cells which is mediated by specific mediators of intercellular signal transduction. This is demonstrated by the fact that treatment of monocytes with IFN- γ or GM-CSF normally up-regulates the expression of such molecules as B7, HLA-DR, ICAM-1 and CD40. In contrast, treatment of monocytes with IFN- γ or GM-CSF with concomitant bridging of the Fc γ RII on monocytes prevents the up-regulation of the B7 molecules without affecting the up-regulation of HLA-DR and ICAM-1. Monocytes are professional antigen presenting cells and Fc γ RII bridging does not affect the up-regulation of HLA-DR on these cells and should therefore not affect their capacity to present antigenic peptides in the context of the HLA-DR molecules. Furthermore, it has been demonstrated that Fc γ RII is capable of mediating phagocytosis of IgG-opsonized erythrocytes (Indik et al., *J. Clin. Invest.* 88:1766 (1991); Tuijnman et al., *Blood* 79:1651 (1992)) and has been shown to be active in endocytosis of human IgG immune complexes (Engelhardt et al., *Eur. J. Immunol.* 21:2227 (1991)). It is therefore proposed that combining the

presence of a specific antigen with an Fc γ RII-bridging agent *in vivo* would result in presentation of antigenic peptides to specific T cells in the absence or reduced presence of the essential co-stimulatory molecules B7-1/2. This treatment will result in modulation of responsiveness of only those T cells able to recognize the specific antigen in question. This finding is of importance for the treatment of diseases where T cells play a crucial role in pathology such as allergic diseases, autoimmune diseases and transplant rejection. The mechanism by which this treatment results in T-cell inactivation is based on the fact that specific antigen presented as bound to or in combination with a Fc γ RII-bridging agent, would result in the internalization of this antigen. Once internalized, the antigen will be degraded into small peptides and these peptides will be presented in the context of class II MHC antigens on the cell surface of the APC. At the same time, proper bridging of Fc γ RII molecules on the APC prevents the up-regulation of B7 co-stimulatory molecules resulting in presentation of the specific antigen to T cells in the absence of the essential co-stimulatory molecules.

In order to prevent transplant rejection, clinicians have experimented with injection of immune cells from the donor prior to the transplantation with the aim of inducing T-cell tolerance or T-cell anergy against the alloantigens. However, instead of desensitization this procedure often resulted in priming against the foreign alloantigens. This priming effect must have been the result of presentation of the alloantigen in the presence of the co-stimulatory molecules B7-1 and B7-2. Given the present invention of specifically preventing the up-regulation of the B7 molecules, it is now possible to treat transplant patients with monocytes from the donor which have been treated with an Fc γ RII-bridging agent. This procedure will result in the induction of donor alloantigen-specific T-cell tolerance.

The following examples serve to illustrate the present invention, but are in no way to be interpreted as being limitative.

Materials and Methods

Monoclonal antibodies

All mAbs were used as purified immunoglobulins. The anti-B7 mAb B7-24 (IgG2a) and anti-CD40 mAb 5D12 (IgG2b) have been previously described (De Boer et al. *Eur. J. Immunol.* 22:3071 (1992)). The anti-ICAM-1 mAb B-C14 was kindly provided by Dr. J.

Wijdenes (Innotherapie Bessançon, France). The mAbs to HLA-DR (PE-labeled), CD20 (PE-labeled), CD3 (FITC-labeled), CD14 (FITC-labeled) and the isotype control antibodies were purchased from Becton and Dickinson (Erembodegem, Belgium). The CTLA-4 human IgG fusion protein was purified from the supernatant of a cell line stably transfected with a cDNA
5 encoding the fusion protein. This cell line was a gift of Dr. A. Lanzavecchia (Basel Institute for Immunology, Basel, Switzerland). Goat anti-human (affinity isolated polyclonal goat F(ab')₂ anti-human IgG FITC-labeled, Tago, CA, USA) was used as second reagent to reveal CTLA-4 IgG staining.

Isolation and culture of monocytes

10 Buffy coats obtained after cytophoresis of healthy donors were used to prepare monocyte cultures. Mononuclear cell suspensions were obtained after buoyant density centrifugation of buffy coats on Lymphoprep (Nycomed, Oslo, Norway). The monocyte-enriched, E-negative, fraction was separated from T lymphocytes by standard roset formation with SRBC followed by Lymphoprep sedimentation. Monocytes were further enriched by the
15 cold aggregation technique. Briefly the cell suspension was allowed to clump by low speed rotation at 4 °C. Cell clumps were separated from the rest of the cells by centrifugation, this population was > 89% CD14⁺. In some experiments monocytes were isolated by adherence to plastic dishes for 30-40 min at 37 °C in a 5% CO₂ atmosphere. After incubation, the non-adherent cells were removed by repeated vigorous washing. The resulting cultures contained
20 at least 80% monocytes and less than 20% of cells as revealed by phenotype analysis (FACS). Monocytes (1x10⁶/ml) were cultured in RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum, non-essential amino acids, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM L-glutamine, 2 mM sodium pyruvate and, 50 µM 2-mercaptoethanol, in the absence or presence of various stimuli or human recombinant cytokines. IFN-γ (100
25 U/ml), GM-CSF (0.8 µg/ml), TNF-α (1000 U/ml), IL-2 (50 U/ml) were added to monocyte cultures for a period of 24 h. Bridging of the FcR was achieved by culturing monocytes on Petri dishes that had been pre-coated for 30 min at 37 °C with standard human IgG (γ globulins), whole molecule or F(ab')₂ fragment as control (ChromPure human IG, Jackson Immuno Research, West Grove, PA) and extensively washed with PBS. After overnight
30 culture the cells were recovered with a cell scraper and counted by Trypan Blue exclusion.

Purification of T cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat by centrifugation on Lymphoprep (Nycomed, Oslo, Norway). T cells were further purified by depletion of monocytes, B cells and NK cells using Lympho-Kwik T (One Lambda, Los Angeles, CA) according to the manufacturers protocol.

Mixed lymphocyte cultures

Purified T cells (1×10^5 /well) were cultured in 96-well round-bottom tissue culture plates (Falcon 3072, Becton Dickinson, Oxnard, CA) in the presence of various numbers of syngeneic or allogeneic monocytes as stimulator cells. After 3 days of culture, cells were pulsed for 16 h with $0.5 \mu\text{Ci}$ [^3H]-Thymidine (specific activity 5 Ci/mMol, Isotopchim, Ganagobie-Peyruis, France), after which the cells were harvested using an automated cell harvester. [^3H]-Thymidine incorporation was determined with a liquid scintillation counter. Proliferation of T cells were performed in triplicate wells. In inhibition experiments monocytes were pre-incubated with mAb B7-24 ($2 \mu\text{g/ml}$ per 10^6 cells) or an isotype control mAb in complete RPMI for 30 min at 4°C before they were dispensed in 96 well round-bottom plates. Bridging the FcR of the monocytes during the MLR was achieved by using 96-wells round-bottom culture plates that had been pre-coated with human IgG, 1 mg/ml , in PBS for 30 min at 37°C and washed three times with cold PBS. Human IgG F(ab')₂ fragment coated plates were used as control.

Antigen-specific proliferation

Peripheral blood mononuclear cells (PBMC) isolated on a Ficoll-Hypaque gradient (density 1.077) (Pharmacia LKB, Uppsala, Sweden) were washed and resuspended in complete medium, consisting of RPMI 1640 (Gibco, Paisley, Scotland) supplemented with penicillin, streptomycin, glutamine and 5% autologous plasma. They were cultured at a concentration of 1×10^6 cells per ml, in 96 well flat bottom culture plates (Falcon, Labware, Lincoln Park, NJ), pre-coated by incubation overnight either with human serum albumin (HSA) (Behringwerke, Marburg, Germany) or with human γ -globulins (Cohn fraction II) (Sigma, St Louis, MO), each at a concentration of $10 \mu\text{g/ml}$ of phosphate buffered saline (PBS), and followed by extensive washing. The following antigens were used, each at an indicated final concentration which in preliminary experiments had been shown to induce

optimal T cell proliferation: Tetanous toxoid (Wyeth, USA) 0.5 Lfu/ml; Varidase (Lederle, Belgium) 100IU/ml; Tuberculin (Statens Serum Institute, Copenhagen, Denmark) 5IU/ml; Cytomegalovirus antigen (Behringwerke) 0.01IU/ml; Herpes simplex virus antigen (Behringwerke) 0.02IU/ml; Varicella antigen (Behringwerke) 0.1U/ml; Mumps antigen (Behringwerke) 1/2000; Influenza virus antigen (Duphar, Belgium) 1/1000; Candida albicans (Haarlem, Allergenen Laboratorium, The Netherlands) 0.5 µg/ml. The following mitogens were also used for stimulation: Phytohemagglutinin (PHA) (Wellcome Diagnostics, Temple Hill Dartford, England) 0.5 µg/ml; Concanavalin A (ConA) (Sigma) 5 µg/ml; and Pokweed Mitogen (PWM) (Calbiochem, La Jolla, CA) 0.5 µg/ml . Cells were cultured in a 5% CO₂ humidified atmosphere for 3 days (PHA, ConA) or 6 days (PWM, antigens). Eight hours after a 1 µCi (³H)-thymidine pulse (Amersham, Buckinghamshire, England), cells were harvested and processed for determination of (³H)-thymidine incorporation in a liquid scintillation counter.

Cytokine measurements

MLR supernatants were taken after 24h culture and tested for the presence of various cytokines. IL-2 production was measured by bioassay using a subclone of the murine CTLL cell line, using recombinant human IL-2 as standard and is expressed as (³H)-thymidine incorporation of the CTLL cells. TNF-α was determined by ELISA (Innotest hTNF-α, Innogenetics, Belgium) with a detection limit of 10 pg/ml. IL-10 was measured by ELISA using two mAbs: B-N10 as coating antibody and biotinylated BT10 as detecting antibody. Recombinant IL-10 was used as a standard, the detection limit of this ELISA is 5 pg/ml. TGF-β was measured in a bioassay using the MV1 Lu cells line . This bioassay has a detection limit of 50 pg/ml.

Prostaglandin E2 measurement

Prostaglandin E2 produced by monocytes during the MLR under various culture conditions was measured using an ELISA system (Boehringer Mannheim, Brussels, Belgium). To test the effect of the prostaglandin synthesis on T cell activation, the cyclooxygenase inhibitor Indomethacin (Sigma) (100 µM) was added at the beginning of the culture.

FACS analysis

For immunofluorescence analysis, purified monocytes (1×10^6) were resuspended in PBS containing 1 % FCS, 0.1 % NaN_3 and 10 % normal rabbit serum. The cells were incubated at 4 °C for 30 min to block FcR-binding sites. The cells were subsequently
5 incubated for 30 min at 4 °C with primary mAbs of the appropriate specificity, washed in PBS containing 1 % FCS and 0.1 % NaN_3 and, incubated for an additional 30 min at 4 °C with FITC-labeled goat anti-mouse Ig (Sigma). The cells were analysis on a FACScan* instrument (Becton Dickinson & Co, Mountain View, CA).

Example 1 : Modulation of B7-1 expression on human monocytes

10 It is known that B7-1 expression on monocytes can be up-regulated by culture in the presence of $\text{IFN-}\gamma$ (Freedman et al. *Cell. Immunol.* 137:429 (1991)). Several cytokines were tested for their capacity to induce B7-1 expression on monocytes. In addition, it was tested whether B7-1 expression could be induced by FcR bridging on monocytes. Primary human monocytes were isolated by the cold aggregation technique and examined for cell surface
15 expression of B7-1 using flow cytometry. Figure 1 shows that freshly isolated monocytes lack detectable B7-1 cell surface protein, when stained with the anti-B7-1 mAb B7-24. However, after 24 h in culture medium alone, low but significant amounts of B7-1 could be detected on their cell surface. Upon culture for 24 h, of the different cytokines tested, not only as previously reported in the literature $\text{IFN-}\gamma$ (Freedman et al. *Cell. Immunol.* 137:429 (1991)),
20 and GM-CSF markedly increased B7-1 expression. Interestingly, FcR bridging by culturing monocytes on human IgG-coated dishes seemed to prevent the spontaneous increase of B7-1 surface expression.

Down-modulation of B7-1 expression on human monocytes could also be observed after incubation with human IgG in solution. However, binding of the Fc part of human IgG
25 by itself was not enough to modulate the expression of B7-1 on the monocytes. Only when the FcR-bound human IgG was subsequently bridged by the addition of a rabbit antiserum against human IgG, the down-modulation of B7-1 could be achieved (Figure 2).

Example 2 : Effects of FcR bridging on monocyte B7-1, CD40, HLA-DR and ICAM-1 expression when activated by IFN- γ or GM-CSF

The experiment described above illustrates that FcR bridging on monocytes prevents spontaneous increase of B7-1 surface expression. It was therefore also tested as described in the legend of Figure 3 and in the section Materials and Methods above, whether bridging of the FcR on monocytes could prevent up-regulation of B7-1, even in the presence of potent inducers such as IFN- γ or GM-CSF. Monocytes were cultured overnight on human IgG-coated dishes before IFN- γ or GM-CSF was added to the cultures. After an additional 24 h culture period, the cells were recovered and analyzed by FACS for expression of different cell surface molecules. As shown in Figure 3, the IgG-treatment had little or no effect on the spontaneous expression level of the cell surface molecules HLA-DR, ICAM-1 and CD40. Interestingly, FcR bridging strongly inhibited the B7-1 and CD40 up-regulation by IFN- γ or GM-CSF. Whereas only a modest effect was observed on the up-regulation of HLA-DR, and ICAM-1 by IFN- γ or GM-CSF.

The effects of FcR bridging on the expression of a number of other molecules on monocytes is summarized in Table 1. Table 1 show that the relative expression levels of B7, ICAM-3 and CD14 show the strongest decrease. The expression of CD11b, CD16, CD48 and CDw50 is only slightly decreased. The expression of CD40, CD43, CD55, CD58, CD59, ICAM-1 and HLA-DR is slightly increased whereas CD44 a stronger up-regulation.

Example 3 : FcR bridging modulate both B7-1 and B7-2 on monocytes

Recently it was reported in the literature that there are two different B7 molecules, named B7-1 and B7-2 (Freeman et al., *Science* 262:909 (1993)). The experiments described above were performed with a monoclonal antibody specific for the B7-1 molecule. The CTLA-4-Ig fusion protein as described in the section Materials and Methods above, is known to recognize both B7-1 and B7-2 on monocytes and other antigen presenting cells (Freeman et al., *Science* 262:909 (1993)). It was therefore also tested, as described in the legend of Figure 4 and in the section Materials and Methods above, whether the expression of the B7-2 molecule on monocytes was influenced by FcR bridging. Figure 4 shows that when monocytes cultured on IgG-coated plates were analyzed for expression of both B7 molecules

using a CTLA-4-Ig fusion protein, it was found that this treatment down-modulated both B7-1 and B7-2.

Example 4 : Effect of FcR bridging on monocytes on the activation of T cells in Mixed Lymphocyte Cultures

5 As bridging of FcR is able to prevent the up-regulation of the B7 molecules on monocytes, it was tested whether this treatment would influence their stimulatory capacity to activate resting T cells. Purified T cells were cultured in the presence of various numbers of syngeneic or allogeneic monocytes in 96-well round-bottom culture plates pre-coated with IgG, so that FcR bridging was persistent during the entire culture period and proliferation
10 was measured by (³H)-Thymidine incorporation. In Figure 5a it is shown that monocytes cultured on IgG-coated plates were much less potent in stimulating T-cell proliferation than untreated monocytes. A mean inhibition of 54 ± 15 % was found in a total of 10 experiments using cells from different individuals. In addition the proliferative response of T cells to autologous monocytes was completely absent in the IgG-coated plates.

15 Cell proliferation measured by [³H]-Thymidine incorporation on day 4 is a delayed measurement of APC-T cell interactions that have occurred much earlier in the MLR. It was therefore also evaluated whether another parameter of T-cell activation, namely IL-2 secretion, was concordant with the proliferative activity under identical culture conditions. We determined the content of IL-2 in the supernatants of the MLR cultures using the CTLL
20 bioassay. Figure 5b shows that untreated monocytes have the stimulatory capacity to induce a strong IL-2 release in the MLR supernatants. In contrast, the supernatants obtained with monocytes isolated and cultured in the presence of coated human IgG did not contain detectable IL-2 activity. These results indicate that bridging of FcR on monocytes greatly affects their capacity to stimulate resting T cells, both at the level of proliferation and
25 lymphokines secretion.

Example 5 : Blocking the B7-1/CD28 interaction is not sufficient to block the activation of T cells in Mixed Lymphocyte Cultures

In view of the importance of the B7-1/CD28 interaction as a co-stimulatory signal the

importance of this interaction for the regulation of alloantigen-mediated T-cell proliferation and IL-2 production was measured. To this, purified T cells were stimulated with allogeneic monocytes in the presence of mAb B7-24, which is capable to block anti-CD3-induced T-cell activation using mouse fibroblasts transfected with human B7-1 (De Boer et al., *Eur. J. Immunol.* 22:3071 (1992)). Figure 6 shows that addition of mAb B7-24 had little or no effect on the T-cell proliferation but significantly decreased the IL-2 release in supernatants. Although these results suggest the importance of the B7-1 molecule on the APC function of monocytes for lymphokine secretion, the blocking of the B7-1/CD28 interaction could not account for the strong inhibition observed in the experiments above, when using IgG-treated monocytes as APC. This means that for complete impairment of the co-stimulatory function of monocytes both B7-1 and B7-2 must be absent, which is achieved by the specific cross linking of FcR as described in this invention.

Example 6 : FcR bridging inhibits antigen-specific proliferation of T cells.

To determine the effect of FcR bridging on the APC function of monocytes to induce antigen-specific proliferation of T cells, purified T cells and monocytes obtained from healthy donors were stimulated as described above in the Materials and Methods section, with a number of different recall antigens. Table 2 shows that FcR bridging on monocytes strongly inhibits their capacity to induce a specific T-cell response to viral, bacterial and fungal antigens, whereas the treatment did not impair the capacity of the T cells to respond to mitogenic stimuli, all measured by (³H)-Thymidine incorporation. These results indicate that specific proliferative responses of human T cells to soluble antigen are markedly reduced or even abolished by specific bridging of FcR on the APC.

Example 7 : The impaired capacity of APC to co-stimulate T cells after FcR bridging is not mediated by the induction of soluble immunosuppressive mediators

FcR bridging on monocytes is known to deliver a very strong activation signal for the release of soluble mediators. It is also known that monocytes can produce potent soluble immunosuppressive factors (Valitutti et al., *Immunology* 67:44 (1989); Paswell et al., *J.*

Immunol. 123:115 (1979)). In order to determine whether the release of soluble immunosuppressive factors play a role in the T-cell un-responsiveness after FcR bridging on monocytes, the content of the MLR supernatants were analyzed by specific immuno assays or bioassays as described in detail in the section Materials and Methods above, for the presence of the well known cytokines IL-10, TGF- β and TNF- α . As shown in Table 3, there was no significant release of TGF- β in MLR supernatants when using IgG-treated monocytes as APC, whereas only small amounts of IL10 were found. In contrast, very large amounts of TNF- α were secreted in the MLR cultures following the FcR bridging. This TNF- α was mainly produced by the monocytes, since control cultures of monocytes only on IgG-coated plates gave about the same TNF- α production as in the presence of T cells. To assess the role of TNF- α as immunosuppressive agent in our experimental system, we added recombinant human TNF- α to a MLR using untreated monocytes as APC. Under those culture conditions, we found no inhibition of the T-cell proliferation or IL-2 release (data not shown). This demonstrates that TNF- α is not involved in the inhibition of T-cell responses induced by the FcR bridging on monocytes.

Lipid mediators derived from cell membranes, such as the prostaglandins, are known to be produced during T cell-macrophages interactions (Coquette et al., *Eur. J. Pharmacol.* 226:1 (1992)). Prostaglandins, particularly prostaglandin E2 (PGE2), modulates the function of immunocompetent cells by suppressing T-cell and macrophage function (Wong et al., *J. Immunol.* 148:2118 (1992); Gallay et al., *J. Immunol.* 150:5086 (1993)). There are many reports that PGE2 biosynthesis can be initiated by cytokines such as IL-1 or TNF- α (Elliott et al., *Growth-factors* 6:15 (1992)). Furthermore, it has been demonstrated that FcR bridging on monocytes can result in the secretion of PGE2 (Finbloom et al., *J. Immunol.* 150:2382 (1993); Singh et al., *J. Immunol.* 151:2786 (1993)). Therefore the PGE2 content in supernatants of MLR cultures was determined using a specific immuno assay as described in the section Materials and Methods described above. Table 4 shows that PGE2 is released in large amounts only in the cultures on IgG-coated plates. In order to investigate the inhibitory effect of PGE2 on the T-cell activation during the MLR, the cyclooxygenase inhibitor indomethacin was added at the beginning of the culture. Although indomethacin effectively prevented PGE2 release, the T-cell proliferation still remained inhibited. This demonstrates that PGE2 was not responsible for the immune suppression observed when monocytes are cultured on human IgG-coated plates.

Example 9 : The impaired capacity of APC to co-stimulate T-cells after FcR bridging with Fc γ RI-deficient monocytes

To investigate which Fc γ -receptors on the monocytes are involved in the down modulation of the B7 molecules and the resulting impaired co-stimulatory capacity, monocytes from an individual known to have a mutation in the gene for the Fc γ RI, resulting in the absence of this molecule on the cell surface, were used to present several antigens as listed in Table 5. Table 5 shows that FcR-bridging on monocytes from an Fc γ RI-deficient individual also strongly inhibits the capacity of these monocytes to induce a specific T-cell response to viral, bacterial and fungal antigens, whereas the treatment did not impair the capacity of the T cells to respond to mitogenic stimuli. These results indicate that if Fc γ RI could mediate the down modulation of the B7-molecules and the resulting impaired co-stimulatory capacity of monocytes, other Fc γ -receptors can do so as well.

Example 10 : The impaired capacity of APC to co-stimulate T cells after FcR bridging can be blocked by specific monoclonal antibodies to Fc γ RII

To further investigate which Fc γ -receptors on the monocytes are involved in the down-modulation of the B7 molecules and the resulting impaired co-stimulatory capacity, monocytes cultured on IgG-coated plates were stimulated with several antigens in the presence of specific monoclonal antibodies directed to the different Fc γ -receptors. The proliferative capacity of T cells added to these cultures was measured by (3 H)-Thymidine incorporation as described in detail in the Materials and Methods section above. Figure 7 shows that only a monoclonal antibody specific for the Fc γ RII can remove the effect of FcR bridging on monocytes. Figure 8 shows that indeed for several antigens tested a monoclonal antibody to the Fc γ RII completely prevents the impairment of monocytes to stimulate T-cell activation after FcR bridging.

Table 1

Cell surface antigen	Relative expression level after FcR bridging
CD11b	-
CD14	---
CD16	-
CD31	○
CD40	+
CD43	+
CD44	++
CD48	-
CDw50	-
CD55	+
CD58	+
CD59	+
ICAM-1	+
ICAM-3	--
B7-1	---
B7-1/2*	---
HLA-DR	+

Table 2 : FcR bridging inhibits antigen-specific proliferation of T cells*

	Expt. 1			Expt. 2		
	HSA(10 μ g/ml)	HGG(1 0 μ g/ml)		HSA(10 μ g/ml)	HGG(1 0 μ g/ml)	
Stimulus	Mean	Mean	%inhib	Mean	Mean	% inhib
PWM+ +	40,127	64,174	-	74,931	79,458	-
PHA+ +	76,658	73,960	-	106,241	93,462	-
ConA+ +	55,817	60,883	-	75,715	70,921	-
Tetanus	76,370	12,100	84	93,067	23,012	75
Candida	38,007	9,904	74	97,209	2,904	97
Varidase	17,199	3,441	80	59,238	1,830	97
CMV	303	419	-	24,002	423	98
Herpes	25,793	3,127	88	84,844	2,268	97
Varicella	40,157	3,509	91	92,281	14,737	84
Mumps	14,472	1,142	92	23,150	631	97
Influenza	6,421	1,032	84	31,211	5,731	82
Tuberculin	10,346	1,135	89	44,987	1,019	98

Table 3 : Release of solubles immunosuppressive mediators after FcR bridging*

	Untr eated mon ocyte s			IgG- treat ed mon ocyte s			F(ab') ₂ - treat ed mon ocyte s		
	IL- 10 ^s (pg/ ml)	TGB -β _{II} (pg/ ml)	TNF- α+ ⁺ (pg/m l)	IL- 10 (pg/ ml)	TGB -β (pg/ ml)	TNF -α (pg/ ml)	IL- 10 (pg/ ml)	TG B- β (pg /ml)	TNF -α (pg/ ml)
Monocytes alone	<	<	183	<	<	1974	<	<	75
Syngeneic MLR	<	<	183	30	<	1934	<	<	84
Allogeneic MLR	<	<	199	28	<	1591	<	<	81

Table 4 : PGE2 production by human monocytes after FcR bridging*

	Untreated monocytes [§]		IgG-treated monocytes		IgG-treated monocytes + indometacin ^π	
	PGE2 + (pg/ml)	Proliferation (CPM)	PGE2 (pg/ml)	Proliferation (CPM)	PGE2 (pg/ml)	Proliferation (CPM)
Monocytes alone	1,810	2,945	14,100	468	1,834	426
Syngeneic MLR	1,632	9,036	9,410	2,184	2,156	1,999
Allogeneic MLR I	2,179	70,504	10,300	47,423	1,623	40,030
Allogeneic MLR II	2,534	75,687	11,000	53,699	1,644	47,270

Table 5 : FcR bridging on monocytes from an Fc γ I-deficient individual results in inhibition of antigen-specific proliferation*

	HSA(10 μ g/ml)	IgG (10 μ g/ml)
Stimulus	Mean	Mean
PWM + +	42,643	78,768
PHA + +	74,193	81,101
Con A + +	52,287	59,975
Tetanus	66,022	14,010
Candida	30,505	11,589
Varidase	14,979	7,690
CMV	730	462
Herpes	20,417	3,451
Varicella	34,952	4,054
Mumps	12,510	965
Influenza	6,090	800
Tuberculin	10,105	2,481

CLAIMS

1. Composition comprising at least one Fc γ RII (CD32) bridging agent, said agent being characterized as impairing the capacity of antigen presenting cells (APCs) to stimulate the activation of antigen-specific T-cells, resulting in antigen-specific T-cell unresponsiveness, with said bridging agent being chosen from the group consisting of:

- aggregated human IgG molecules;
- aggregated Fc fragments of human IgG molecules;
- a bivalent monoclonal antibody to the Fc γ RII;
- a multivalent monoclonal antibody to the Fc γ RII;
- a functionally active fragment of said bivalent or multivalent monoclonal antibody;
- a recombinant fusion protein of 2 or more human IgG Fc parts; or,
- a liposome vesicle comprising any of the foregoing bridging agents.

2. Composition according to claim 1, further characterized in that said Fc γ RII bridging agent prevents the expression of B7-1/2 molecules by these APCs.

3. Composition according to any of claims 1 or 2, further characterized in that said Fc γ RII bridging agent causes the down modulation of ICAM-3 molecules expression by these APCs.

4. Composition according to any of claims 1 to 3, wherein said Fc γ RII bridging agent is contained in liposome vesicles.

5. Composition according to any of claims 1 or 4, further characterized in that said composition comprises a specific antigen or antigen-complex combined with said Fc γ RII bridging agent.

6. Composition according to claim 5, further characterized in that said specific antigen is a specific alloantigen, an autoantigen, or an allergen.

7. Composition according to any of claims 1 to 6, further characterized in that said

Fc γ RII bridging agent consists of aggregated human IgG molecules, or aggregated Fc containing fragments of human IgG molecules.

- 5 8. Composition according to any of claims 1 to 6, further characterized in that said Fc γ RII bridging agent consists of a bivalent or multivalent Fc γ RII specific monoclonal antibody, or a functionally active fragment of said antibody.
9. Composition according to any of claims 1 to 6, further characterized in that said Fc γ RII bridging agent consists of a recombinant fusion protein of two or more human IgG Fc parts.
- 10 10. Composition according to any of claims 1 to 6, further characterized in that said bridging agent consists of a liposome vesicle comprising any of the bridging agents of claim 1, further characterized in that the Fc regions of said bridging agent are on the outside of the liposome vesicle, and in case of a specific antigen or antigen-complex being added according to claim 5, said antigen or antigen-complex being on the inside of said liposome vesicle.
- 15 11. Method for preparing a composition according to any of claims 1 to 10
12. Composition according to any of claims 1 to 10 for use as a medicament.
13. Composition according to any of claims 1 to 10 for use in therapy or prophylaxis, more particularly for treating or preventing T-cell mediated diseases.
- 20 14. Composition according to claim 13 for modulation of antigen-specific T-cell responsiveness.
15. Composition according to claim 13 for treating allergic diseases.
16. Composition according to claim 13 for treating rejection of solid organs, tissues and cells after transplantation.

17. Composition according to claim 13 for the treatment of autoimmune diseases.
18. Composition according to claim 16 for inducing T-cell anergy.
19. Composition according to claim 16 for inducing T-cell tolerance.
20. Composition according to any of claims 12 to 19, with said composition also
5 comprising immunosuppressive drugs and/or anti-inflammatory drugs such as cyclosporin A, cyclooxygenase inhibitors or corticosteroids.
21. Composition according to any of claims 12 to 19, with said composition also comprising immunomodulatory cytokines.
22. Use of a composition according to any of claims 1 to 21 for the preparation of a
10 medicament for treating or preventing T-cell mediated diseases.
23. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for modulating antigen-specific T-cell responsiveness.
24. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for inducing T-cell anergy.
- 15 25. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for inducing T-cell tolerance
26. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for treating allergic diseases.
- 20 27. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for preventing or treating rejection of solid organs, tissues or cells after transplantation.

28. Use of a composition according to any of claims 1 to 21 for the preparation of a medicament for the treatment of autoimmune diseases.
29. Medicament comprising as an active principle a composition according to any of claims 1 to 21.
- 5 30. Fc γ RII bridged professional antigen presenting cells obtainable by bridging professional antigen presenting cells with any of the Fc γ RII bridging agents or compositions according to any of claims 1 to 21.
31. Therapeutic composition comprising Fc γ RII bridged professional antigen presenting cells according to claim 30.
- 10 32. Fc γ RII bridged professional antigen presenting cells according to claim 30 for use as a medicament, more particularly for treating any of the diseases mentioned in claims 22 to 28.
33. Use of Fc γ RII bridged professional antigen presenting cells according to claim 30 for the preparation of a medicament for treating any of the diseases mentioned in claims 22 to 15 28.
34. Method for producing Fc γ RII bridged professional antigen presenting cells comprising the step of bridging antigen presenting cells with any of the Fc γ RII bridging agents or compositions according to any of claims 1 to 21.
- 20 35. Method for screening for new Fc γ RII bridging agents, with said agent being characterized in that it prevents the B7 molecule expression on professional APCs and/or down-modulates the ICAM-3 expression on professional APCs comprising the step of incubating professional APCs in the absence or presence of the possible Fc γ RII bridging agent and measuring the amount of B7-1/2 and/or ICAM-3 expression.

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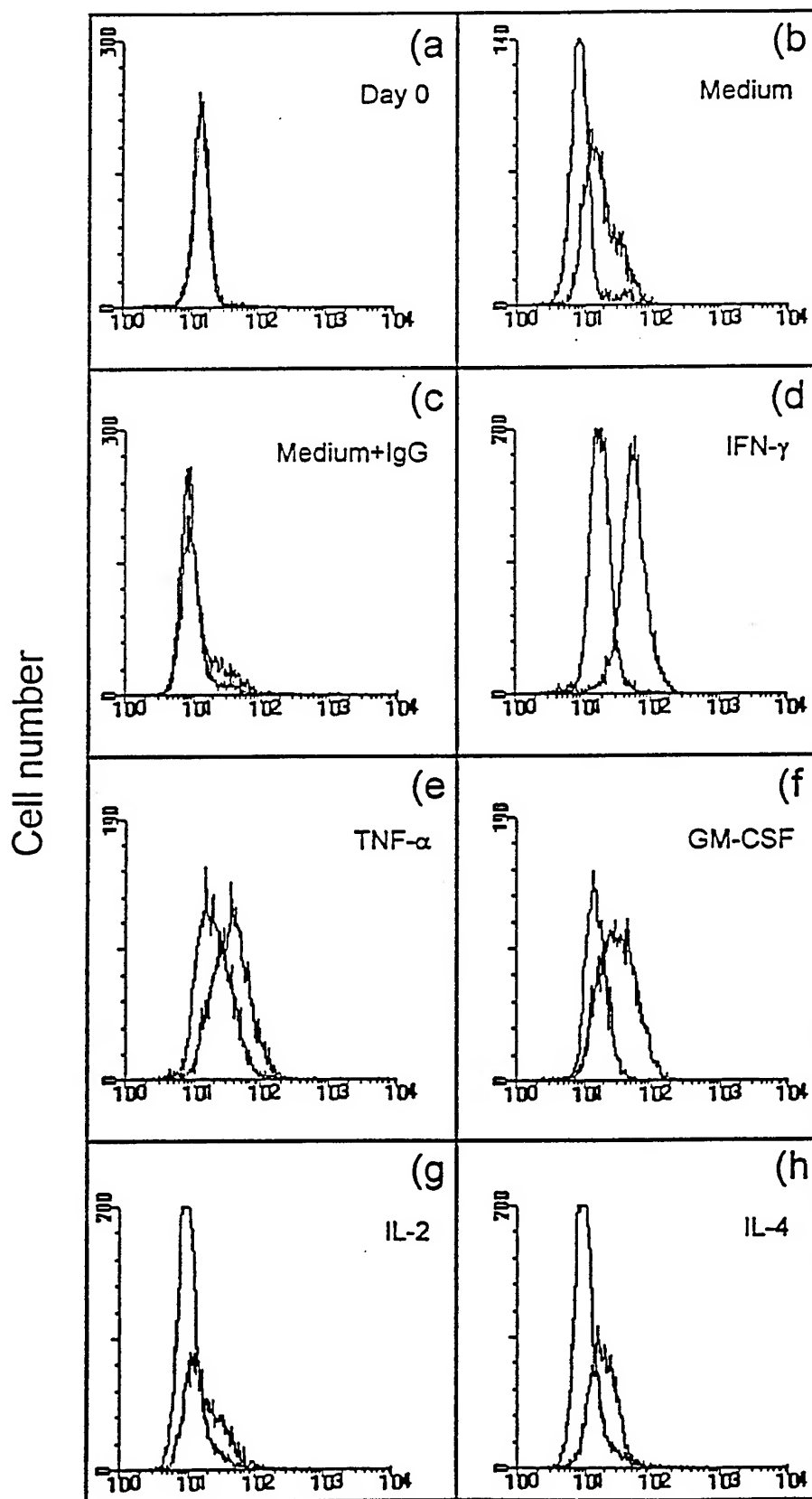


Fig.1

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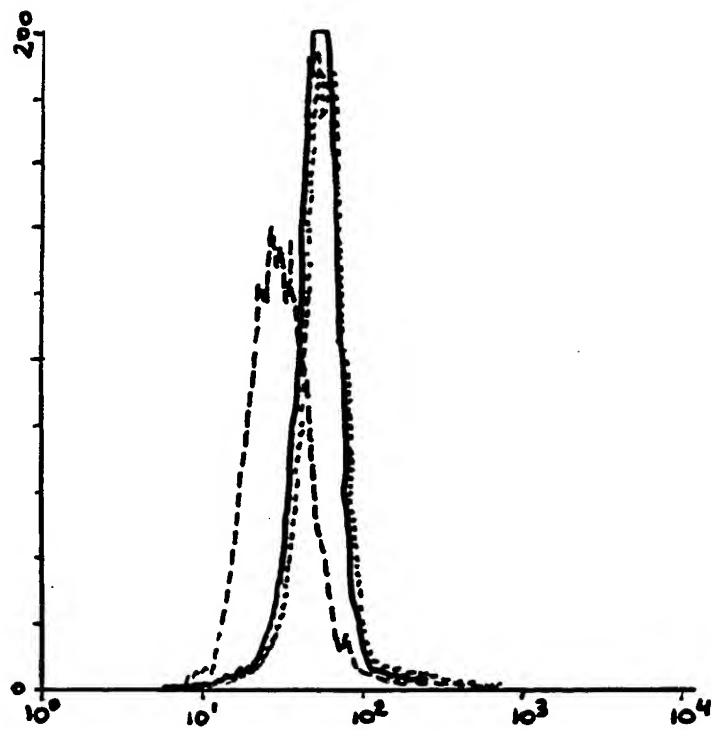
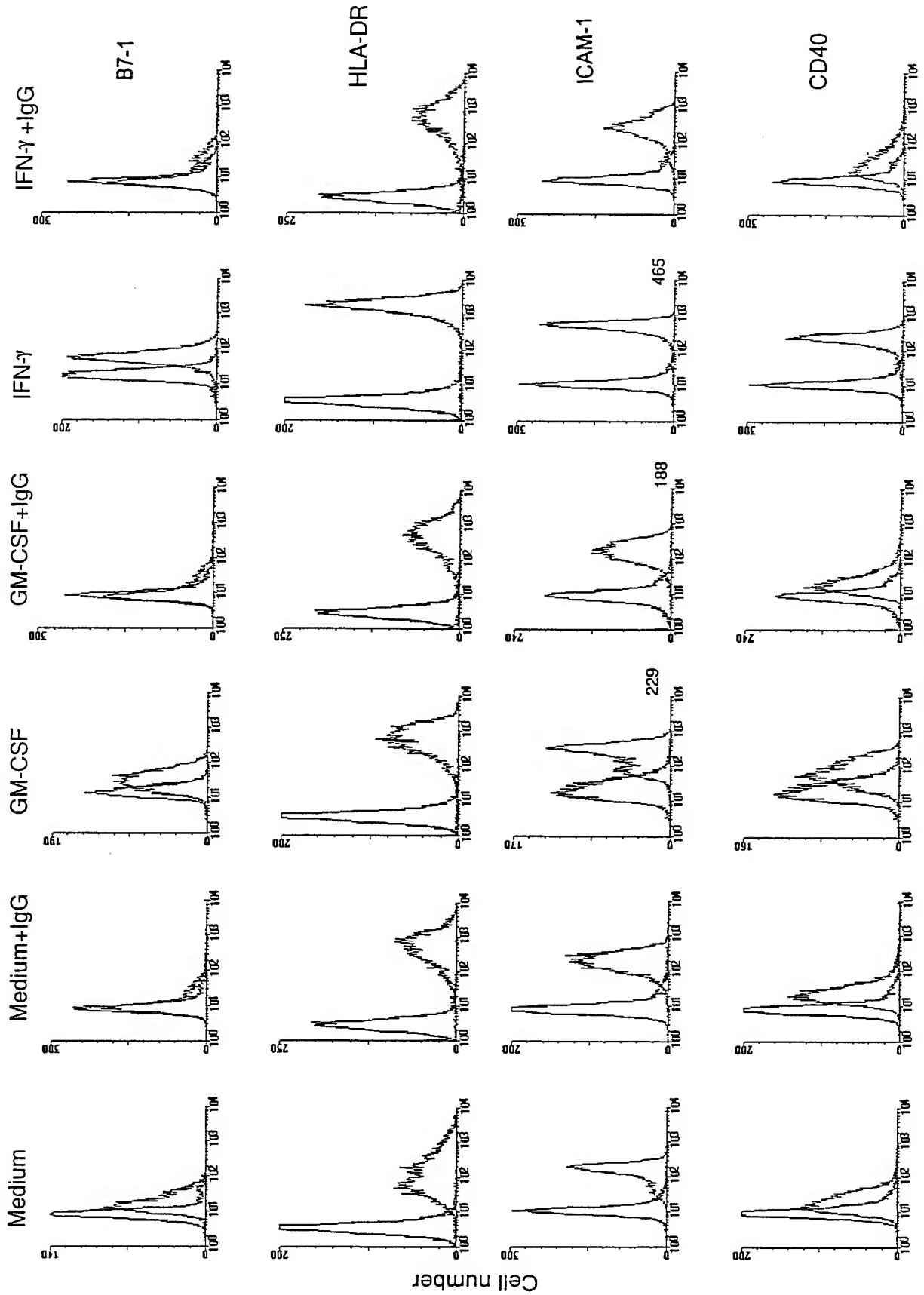


Fig. 2

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Log of fluorescence intensity

Fig. 3

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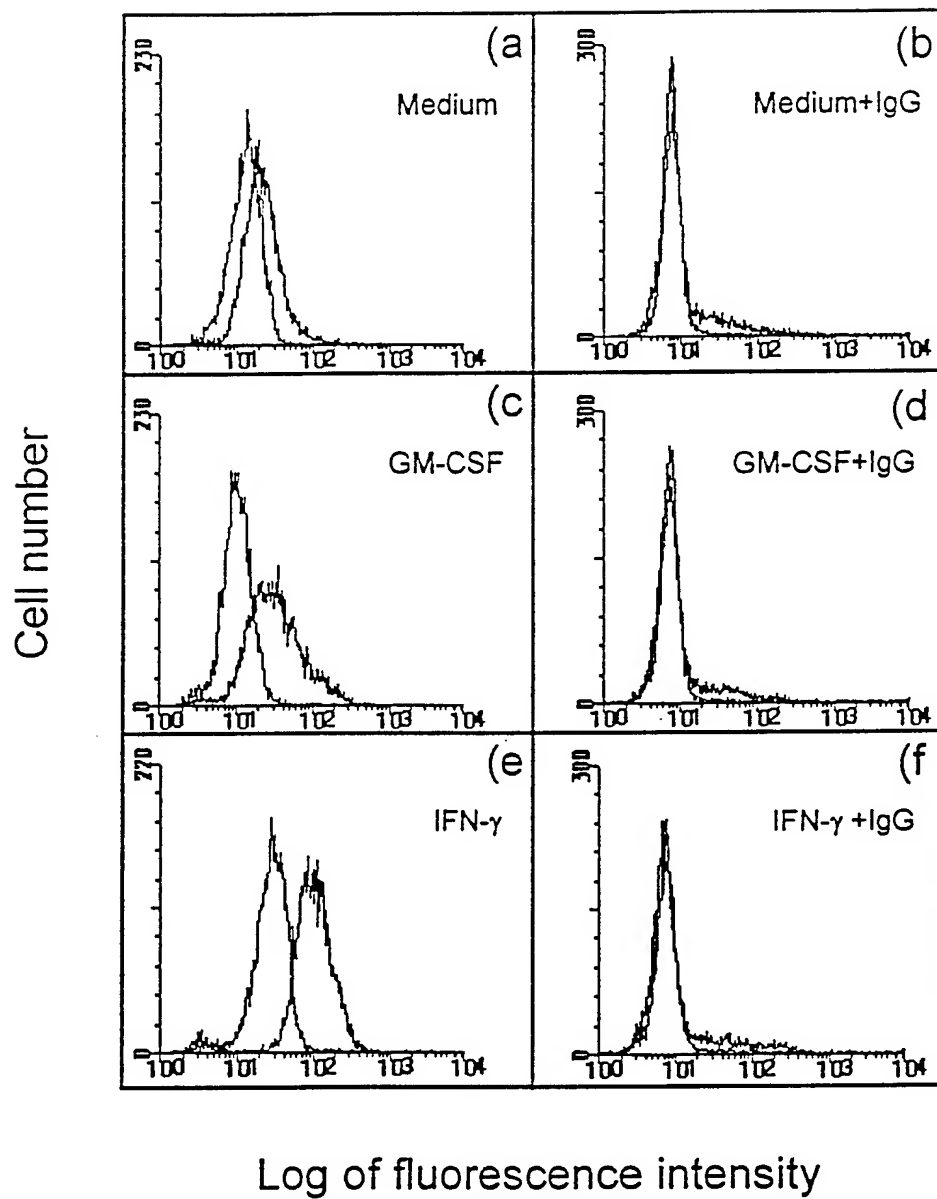


Fig. 4

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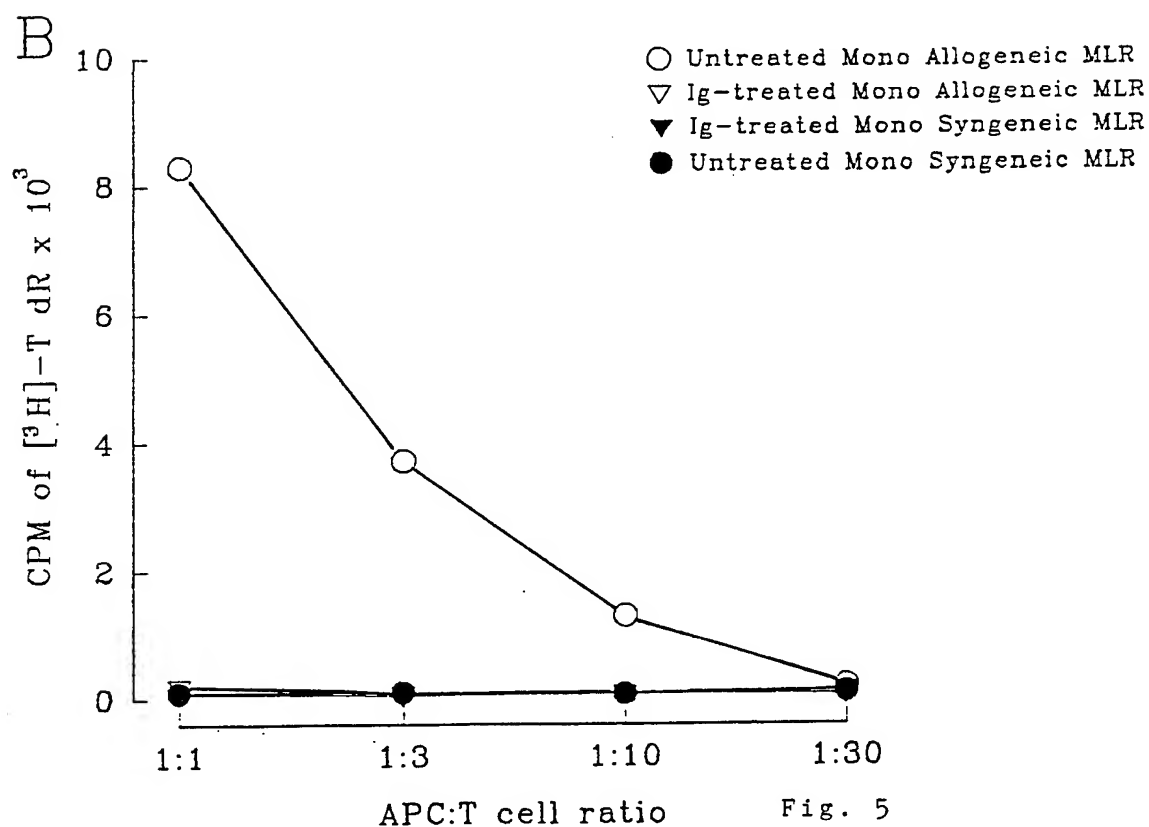
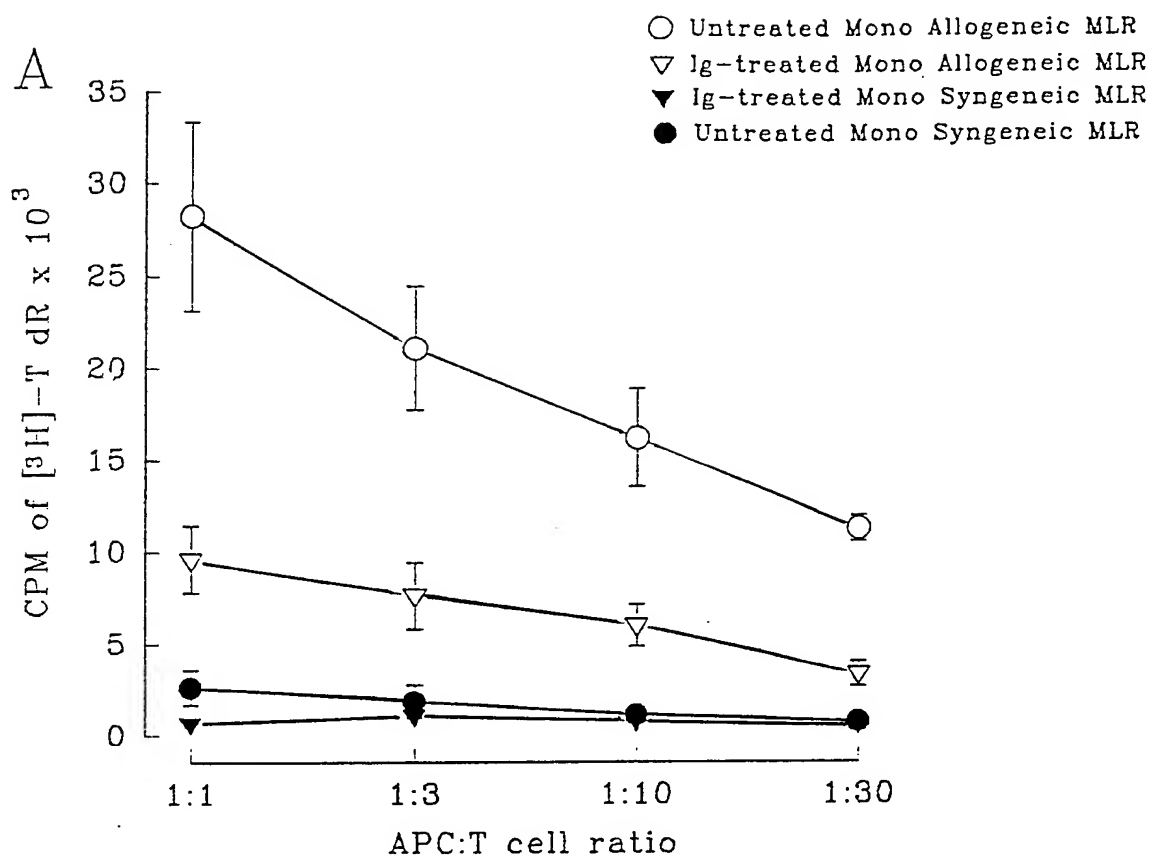


Fig. 5

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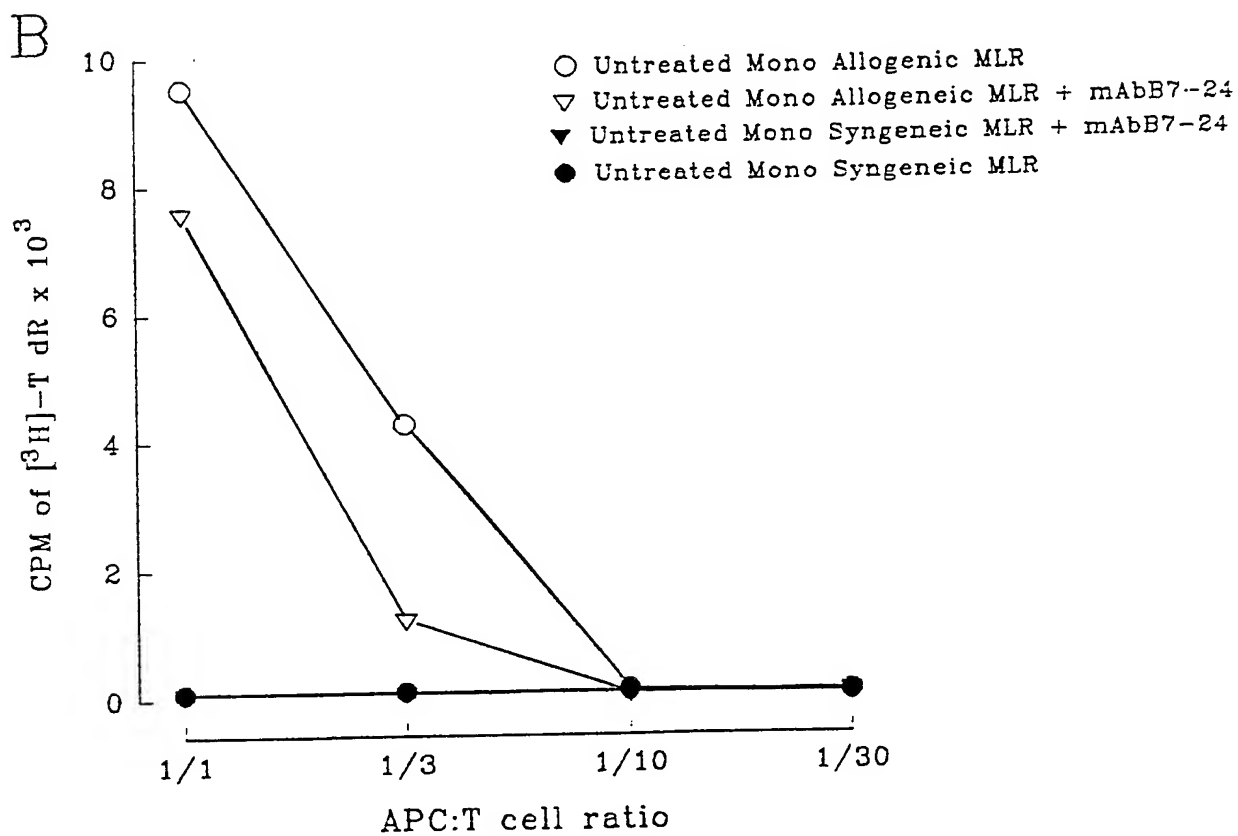
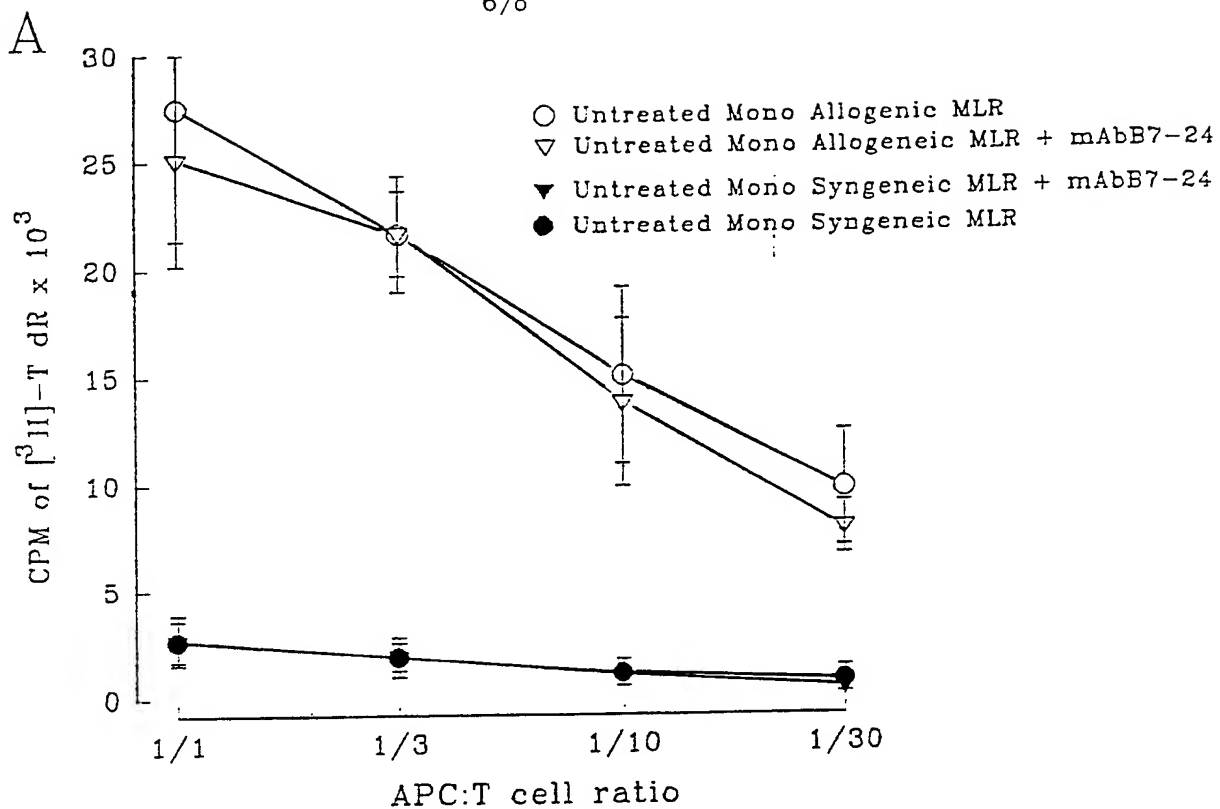


Fig. 6

Figure 7

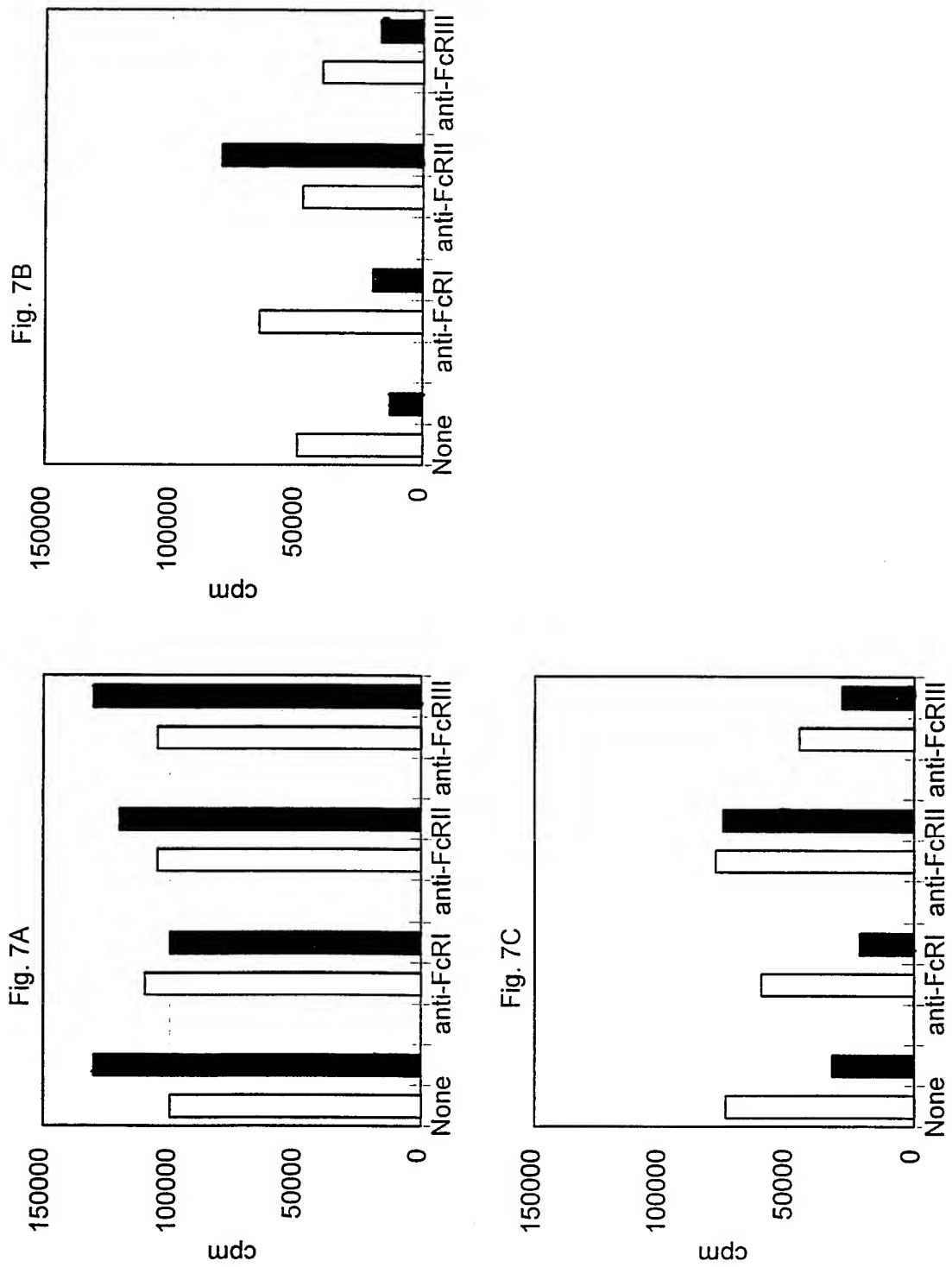
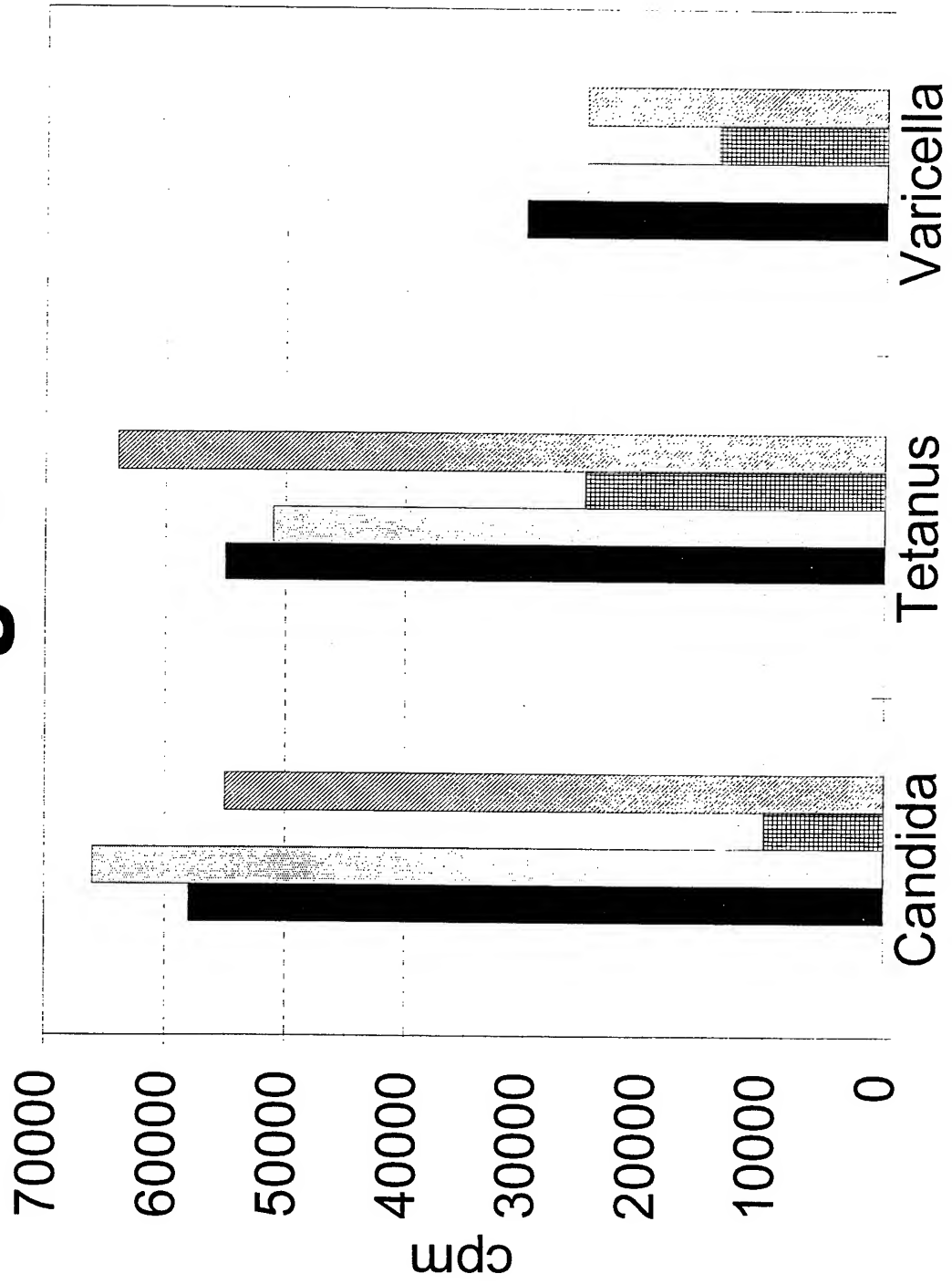


Fig. 8



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/02012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K35/14 A61K9/127 C12N5/08 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF IMMUNOLOGY, vol. 150, no. 5, 1 March 1993 BALTIMORE MD, USA, pages 1794-1803, F. IERINO ET AL. 'Mapping epitopes of human Fcγ₁RII (CDw32) with monoclonal antibodies and recombinant receptors.' cited in the application see abstract see page 1795, left column, line 23 - line 27</p> <p style="text-align: center;">--- -/--</p>	1-35

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

1 September 1995

Date of mailing of the international search report

11. 09. 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
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Nooij, F

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02012

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A	<p>NATURE, vol. 356, no. 6370, 16 April 1992 LONDON, GB, pages 607-609, F. HARDING ET AL. 'CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.' cited in the application see page 609, right column, line 8 - line 26 ---</p>	1-35
A	<p>THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, no. 5, 1 November 1993 NEW YORK NY, USA, pages 1753-1763, V. BOUSSIOTIS ET AL. 'B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance.' cited in the application see abstract ---</p>	1-35
A	<p>EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 22, no. 12, December 1992 WEINHEIM, GERMANY, pages 3071-3075, M. DE BOER ET AL. 'Functional characterization of a novel anti-B7 monoclonal antibody.' cited in the application see abstract ---</p>	1-35
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1 8 A	<p>WO-A-91 16354 (3I RESEARCH EXPLOITATION LIMITED) 31 October 1991 see figure 4 ---</p>	1-35

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02012

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A	US-A-4 579 840 (HAHN) 1 April 1986 cited in the application see column 4, line 10 - line 23 ---	1-35
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Information on patent family members

International Application No

PCT/EP 95/02012

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